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Direct Detection of Drugs of Abuse in Whole Hemolysed Postmortem Blood and Qualitative Measurement in EDTA – Plasma using the CEDIA DAU Urine Assays

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Abstract

The direct and simple detection of a broad spectrum of drugs in whole hemolysed postmortem blood (Part A) and the possibility of qualitative measurement in serum and whole blood using the cloned enzyme donor immunoassay technique (CEDIA) (Part B) is described.
We measured the samples for the presence of amphetamines (AMP), barbiturates (BARB), benzodiazepines (BENZ), cannabinoids, cocaine, LSD, methadone and morphine and its derivates. The confirmation was done by HPLC or GC-MS depending on the drug group.

Part A: An extract of postmortem blood that was not pretreated was measured with the CEDIA DAU® urine system for the above mentioned drugs of abuse. The measurements were repeated with reduced and diluted probe sample and a $ZnSO_4$ protein – precipitation. We showed that this method is very sensitive in toxic and therapeutic drug concentrations for Amphetamines, Barbiturates, Benzodiazepines, Cannabinoids, Cocaine and Morphine. Our recommendation is to dilute the samples 1:1 with water. The only exception is the measurement for amphetamines where you have to do a protein precipitation with $ZnSO_4$ – solution. With barbiturates and LSD we had very few positive samples so we could not do any statement.

Part B: We could verify the thesis of qualitative measurement of drugs of abuse in unpretreated EDTA – plasma using the urine assays. Within 250 EDTA – plasma samples we had seven false – positives (2.8%) and one false – negative (0.4%). The quantitative correlation is only reliable for high substrate – specific substances, like benzoylecgonine, the main cocaine metabolite. For all other measured substances we were able to find a good qualitative correlation using the unpretreated sample.

The CEDIA is very useful in forensic cases as a reliable screening method to identify the samples where a complicated chromatographic quantitation is appropriate.

Key words: *Forensic sciences, Cloned enzyme donor immunoassay; direct detection of drugs of abuse; EDTA - plasma*

1. Introduction

Immunoassay procedures developed primarily for use with urine samples have been successfully adapted to the analysis of whole blood by a number of investigators, but they all used pretreated blood for their analysis. Various methods of pretreatment were developed such as protein precipitation e.g. with methanol (1),(2),(3),(4), acetone (5),(6),(7), trichloroacetic acid (8) or zinc sulfate/methanol (9). Another possibility was the extraction of the blood sample (10) e.g. with butylchloride (11) or the dilution with saline(12). The modifying of the serum, plasma or postmortem blood with N,N-Dimethylformamide(13) was also done. All these pretreatments reduce the advantages of the direct immunoassays - the low cost and quick method of drug detection with a small amount of specimen. In addition it has not been proven whether there is a loss of sensitivity with pretreatment or not.

The number of cases where only a blood sample is available is steadily increasing. This is due to the fact that suspected car drivers or other motorists in Germany can refuse to supply a urine sample, but not a blood sample. That was the reason to investigate a reliable and fast screening method to select those samples where a time consumptive chromatographic quantitation is necessary. According to the studies of Iwersen et al. (14) we proofed the

possibility of direct analysis of untreated blood samples from the institute's routine using the CEDIA DAU® immunoassay.

2. Materials and Methods

2.1 CEDIA

The CEDIA-DAU (cloned enzyme donor immunoassay) is a homogenous immunoassay based on the bacterial enzyme ß-galactosidase of E.coli. which has been genetically engineered into two inactive fragments. These two fragments of the enzyme recombine to the active enzyme. Drug in the sample competes with drug conjugated to one of the inactive enzyme fragments for an antibody-binding site. If there is drug in the sample it will bind to the antibody and leave the drug conjugated enzyme fragment free. This fragment binds to the other unit of the enzyme and produces the active enzyme. The amount of the active enzyme, that generates a color change, is proportional to the antigenic concentration (15). The measurement is done photometrically at 660/570 nm. For all screenings we used the CEDIA® reagents from Microgenics Corporation on a Hitachi 911 from Boehringer, Mannheim Germany.

We measured 250 EDTA-plasma and 25 postmortem blood samples for the presence of Amphetamines, Benzodiazepines, Barbiturates, Cannabinoids, Cocain, Methadon, Opiates and LSD. According to the group specificity of the antibodies the measurements are always measurements for drug groups and not single substances. We did not include Phencyclidine (PCP) in this study because it is not current in Germany.

2.2 Confirmation

We compared the results of the CEDIA – screening with results of the HPLC (High Pressure Liquid Chromatography) or GC-MS (Gas Chromatography-Mass Spectrometry) depending on the drug group using our own confirmation methodologies. Benzodiazepines, Barbiturates and LSD were confirmed by HPLC, Amphetamines, Cannabinoids, Cocain, Methadon and Morphine by GC-MS. We measured all postmortem blood samples for the presence of all the eight drug groups. For the question of quantitative or semiquantitative use of the immunoassay we confirmed only the CEDIA positive samples for the screened positive drug group.

2.3 GC-MS

For the verification of the immunoassay results for Amphetamines, Opiates, Cocaine, Cannabinoids and Methadone we used the gas chromatography/mass spectrometry (GC-MS). This was done for Amphetamines, Cocaine and Opiates with a solid phase extraction on a XAD-column (Detectabuse™, extraction column type R, Biochemical Diagnostics, Inc., New York, USA). For Methadone we used a liquid-liquid extraction with Ether/EtAc 1:1. All the procedures are in accordance to the working manuals of the Institute for Legal Medicine, Munich (16). Measurings were done on a HP 5971 Mass Selective Detector 5890 Series Gas Chromatograph Hewlett Packard, Waldbronn, Germany).

2.4 HPLC

To verify Benzodiazepines, Barbiturates and LSD we used the high pressure liquid chromatography (HPLC). For Benzodiazepines and Barbiturates we used liquid-liquid extraction with Chlorobutane. The HPLC system consisted a HPLC RP-select B column (E. Merck, Darmstadt, Germany) and a diode array detector (HP Series 1100). LSD extraction was done with a solid phase of LSD Immun Elute – columns (Microgenics Corp., Pleasanton, USA).

We use a fluorescence detector stimulating at 320nm and measuring at 420nm with a RP-select – column (E. Merck, Darmstadt, Germany) according to the institute's working manuals (17).

2.5 Limit of Detection

As decision limit we used the detection limit as described by Hallbach et a.l.(18). Thirty blank EDTA – plasma samples and thirty blank postmortem blood samples were screened by CEDIA. The cut-off was calculated for each specimen by adding three standard deviations to the arithmetic median. The cut-offs used in this study are shown in table 1.

2.6 Probe Quality of the measured EDTA-samples

Some of the EDTA-plasma samples had different grades of turbidity. Bye visual observation we obtained four grades of turbidity:

0 no turbidity
1 weak turbidity
2 medium turbidity
3 strong turbidity

Because of the photometer's huge range (3.2A), it was possible to measure also hemolytic samples. We tried to figure out if the hemoglobin in the sample will result in high background absorbance levels (19) or error messages (9).

2.7 General

Results were defined as false-negative if screening results were below the screening cut-off levels of the immunoassay (see Table 1) on postmortem blood samples that contain the target drug above GC-MS or HPLC cut-off concentrations (20).

False-positive was defined as measured with a result higher than the cut-off levels on the immunoassay and not confirmable with GC-MS or HPLC depending on the drug group.

Sensitivity of the CEDIA assay was calculated by using gas chromatography-mass spectrometry as the accepted reference standard . The sensitivity was computed as the number of true positive results divided by the sum of true positives plus false-negatives.

Table 1 : Use of detection limit as decision limit (cut-off)

Drug group	EDTA-Plasma [ng/ml]	postmortem blood [ng/ml]
Amphetamines	35	35
Barbiturates	1000	1000
Benzodiazepines	20	20
Cannabinoids	20	20
Cocaine	50	100
LSD	0,2	0,2
Methadone	35	35
Opiates	20	20

3. PART A: Direct Detection in whole hemolysed postmortem blood

3.1 Pretreatment of the hemolysed postmortem blood samples

We measured the postmortem blood samples first without any pretreatment. Then, we screened all samples with a reduced sample volume. This was one third of the normal sample volume i.e. 1 instead of 3 µl, 2 instead of 6µl or 4 instead of 12µl (21).

In addition, we diluted the samples 1:1 with H_20 and used them with the normal sample volume and again with the reduced sample volume. For a fourth comparison with the results of the unattended sample, we did a protein precipitation with a 3.5% zinc sulfate in 50% methanol as described by Simonick and Watts (9).

3.2 Results of the postmortem blood screening

3.2.1 Benzodiazepines

The results of the comparison between unpretreated postmortem blood, measurements with a reduced, 1:1 with H_20 diluted, 1:1 with H_20 diluted and reduced volume and the $ZnSO_4$-precipitation are shown in Figure 1 for Benzodiazepines. We included in that figure only the 15 results which where different from zero in the confirmation.

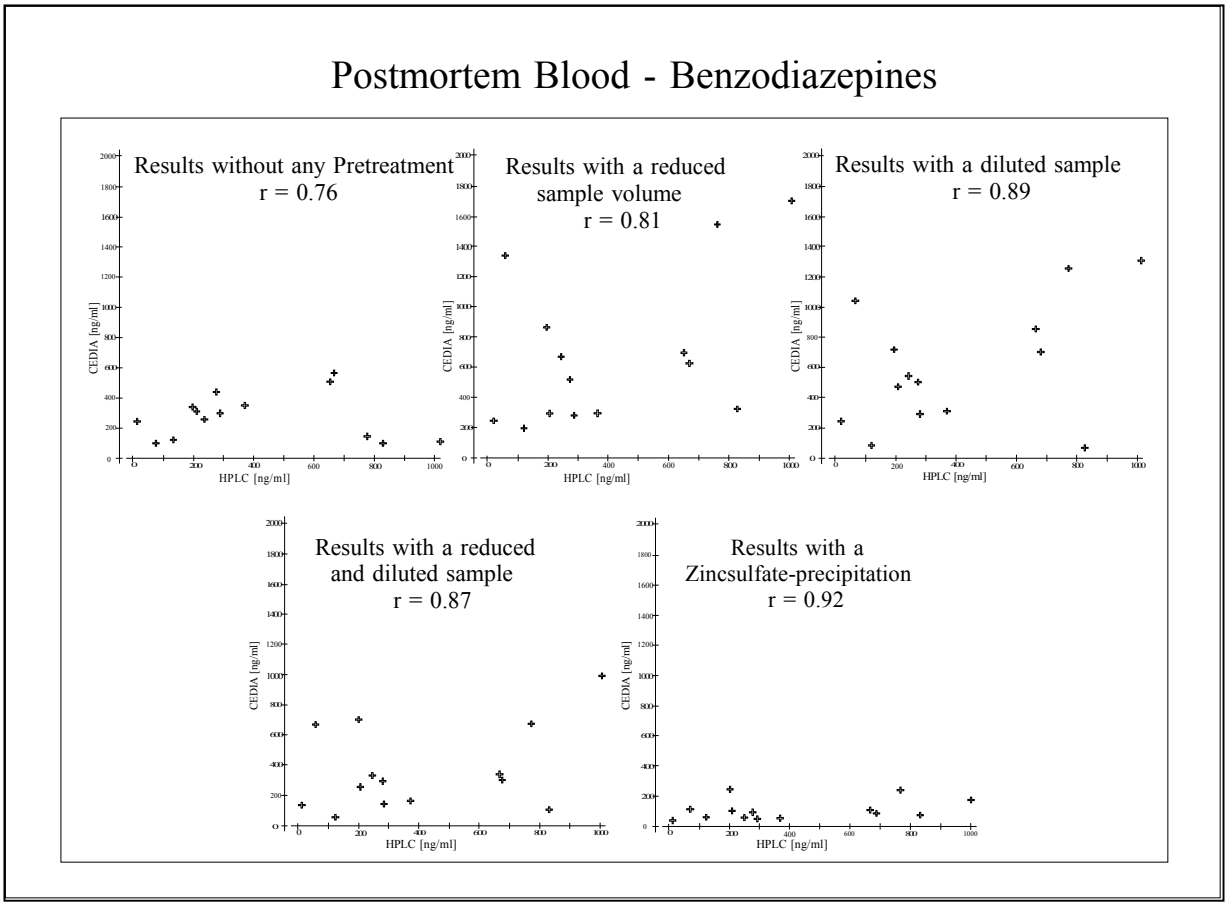

Figure 1: Comparison of the different pretreatings of postmortem blood

As it is shown the best correlations between CEDIA and HPLC for benzodiazepines are those where we diluted with water or made a protein precipitation of the postmortem blood sample. The correlations for benzodiazepines are 0.78 with the undiluted postmortem samples, 0.81 with the reduced volume, 0.89 with the 1:1 with H_20 diluted, 0.87 with the diluted and reduced and 0.92 with the $ZnS0_4$ – precipitation. Within the 25 samples we measured each with the described 5 different methods we had three false – positive samples with concentrations of 50, 45 and 29 ng/ml with the CEDIA assay when using the unpretreated blood sample. These samples could not be confirmed by HPLC. There were no false-negative samples.

Using the $ZnSO_4$ – protein precipitation all our CEDIA – results where below 200ng/ml although the range of the HPLC quantitation was up to 1000 ng/ml. This was probably caused by interference of the immunoassay by the protein precipitation, but nonetheless all positive samples could be detected.

3.2.2 Cocaine

With cocaine we had the best correlations between CEDIA and HPLC with the reduced probe volume (0.94), the diluted sample (0.92) and the protein precipitation (0.92). The undiluted samples had a correlation with HPLC of 0.78 and the diluted samples with a reduced probe volume had 0.90. But of the group of 125 measurements we had five false-positives and six false-negatives. Three of the false-negative results were obtained while measuring with the unpretreated blood, one with the diluted probe, one with the reduced volume and one with the reduced volume and the diluted sample. At all other measurements the results of these samples were above the cut-off levels. The concentrations of the three samples were 439 ng/ml and 80 ng/ml benzoylecgonine and the other sample had a concentration of 15 ng/ml. This gives us a sensitivity of 76% as shown in Table 2.

Table 2: Sensitivities and Specifities of the postmortem blood screening

Method	**Drug**		**CEDIA** positive	negative	**Sensitivtiy**	**Specificity**
HPLC	Benzodiazepines	positive negative	80 5	0 40	100%	89%
	Barbiturates	positive negative	0 0	0 125	----	----
	LSD	positive negative	0 3	0 122	---	98%
GC-MS	Amphetamines	positive negative	0 45	0 80	----	64%
	Cannabinoids	positive negative	26 3	6 90	81%	97%
	Cocaine	positive negative	19 5	6 99	76%	95%
	Methadone	positive negative	4 0	1 120	80%	100%
	Morphine	positive negative	93 5	2 25	98%	83%

3.2.3 Opiates

With the opiates we got the best results with the reduced probe volume (corr. = 0.77), the diluted samples with reduced probe volume and the $ZnS0_4$ – protein precipitation (corr. = 0.75). The other test methods brought us correlations of 0.51 with the undiluted and 0.63 with the diluted samples. We got one false-positive from the 125 measurements were we could not confirm any free opiates. We had two false-negatives one of which contained 4 ng/ml Codeine and 72 ng/ml Morphine and the other 6 ng/ml Dihydromorphine, 12 ng/ml Codeine and 65 ng/ml Morphine. The false-negatives only showed up while measuring with the diluted samples and a reduced probe volume. But they were detectable with the other four methods, which brought us the following results: 189/161 ng/ml unpretreated sample, 132/84 ng/ml with the reduced probe volume, 136/96 with the diluted sample and 131/122 ng/ml with the $ZnSO_4$ -protein precipitation.

3.2.4 Cannabinoids

Measuring the 25 samples for the presence of THC we had six false – negative results using the unpretreated sample. These problems did not reoccur after diluting the samples 1:1 with water. Two other samples produced a false – positive result of 27.4 ng/ml and 55 ng/ml when measuring the diluted sample.

3.2.5 Amphetamines

The measurement of the 25 Amphetamine samples gave us a large (n = 10) number of false-positives: 40%. This may be caused by the cross-reactivity with many sympathomimetic amines (22). Another explanation may be the generation of phenylethylamine by decarboxylation of phenylalanine caused by putrefaction of the postmortem blood (23). Similar numbers of false-positives are also described elsewhere (24) and with other technologies such as Emit. It may be necessary to oxidize and eliminate the cross-reacting sympathomimetic amines with treatment of sodium periodate (22). In our laboratory we measure the samples diluted 1:1 with water, but we cannot exclude the possibility of false-positive results, so we remeasure only those samples, which have suspect to be really positive for amphetamines.

To exclude false-negative results it is necessary to precipitate the proteins with a methanolic $ZnSO_4$-solution (9).

3.3 Conclusions of Part A

Overall we measured 25 samples unpretreated and with 4 pretreatment methods as described above for 8 different drug groups. Of these 1,000 results, we had 6 false-negatives without any error message of the equipment's software. This is probably based on insufficient filling of the sample needle caused by high viscosity of the sample or clots. After diluting the sample with water or reducing the probe volume, no such problems reoccurred.

But we had two times the fact that after diluting and insetting a reduced probe volume there was no possibility to detect a very low concentration of drugs obviously caused by the small volume and diluted concentration.

The sensitivities and specifities of the used assays are shown in table 2.

After this study we can consider that it is sufficient even for the screening of postmortem blood, to dilute the samples 1:1 with water. The only exception is the measurement for Amphetamines where you have to do a protein precipitation with a methanolic $ZnSO_4$ – solution.

4. Part B: Results of the qualitative measurements

4.1 Benzodiazepines

Comparing the results we obtained from CEDIA and HPLC for benzodiazepines we could find a huge range of fluctuation for all measured scales (see Figure 2). The CEDIA – benzodiazepine assay is calibrated for nitrazepam. Depending on the broad spectrum of benzodiazepines and the different cross-reactivities, there is no possibility of doing a quantitative statement. It is not even possible to do an assessment of the drug concentration of the various benzodiazepines contained in the sample.

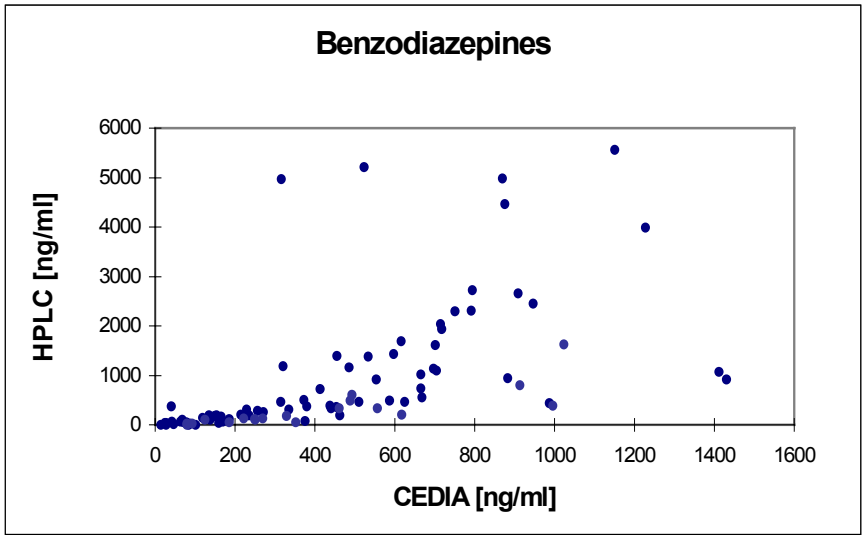

Figure 2 : CEDIA Benzodiazepine Assay in comparison with HPLC

4.2 Amphetamines

In contrast to the other drug groups we had a large matrix effect measuring amphetamines using whole blood and serum. To avoid these matrix effects it is necessary to carry out a pretreatment of the sample. Protein precipitation with organic solvents is very problematic because of the volatile nature of amphetamines and the dry-down step of the organic supernatant (25). Methanolic HCl addition prevents this loss but causes a loss of 30% of cannabinoids (26). Protein precipitation as described e.g. by Simonick and Watts (9) may be a good alternative. Some results of the amphetamine measurements are shown in Table 3. Not surprisingly we did not find pure Amphetamine but also the cross – reacting substances MDMA and MDEA, according to immunoassay's group specificity.

Table 3: Excerpt of the amphetamine screening and confirmation results

No.	Hitachi		MS		
	Serum	ZnSO4	Amph.	MDMA	MDEA
	[ng/ml]		[ng/ml]		
1	0	93	32		
2	33	251	200		
3	0	80		148	
4	0	59			602
5	9	122		526	423
6	405	335	16500	80	

4.3 Cocaine

The comparison between CEDIA and GC-MS measurements for cocaine and its metabolism products gave a good correlation (r = 0.924). We had no false-positive result but, did obtain one false-negative. The concentration of cocaine in this case was 0.6 ng/ml with a concentration of benzoylecgonine (BZE) of 27 ng/ml. It was a sample with a turbidity grade by visual observation of 0. Normally we would not have remeasured this sample, but the extinction procession was suspicious, so we remeasured it, and it was found false-negative.

If we look at the samples where we could only find BZE, the main metabolism product in the confirmation analysis, we get even better results. The correlation is r = 0,97 and the regression equation y = 0.97x + 10.7 in a range from 40 to 2,000 µg/l (see Figure 3). We had one sample with the CEDIA-result of 2,770 µg/l that brought a GC-MS result of 6,630 µg/ml. This huge aberration is probably caused by the immunoassay's saturation in higher concentrations of the drug although the manufacturer declares a measuring range from 0 to 5,000 µg/l (27).

This shows the possibility of quantitative measurement with CEDIA in cases where only one drug is present and the drug specificity is high. Here the cross-reactivity for BZE is 100% (27).

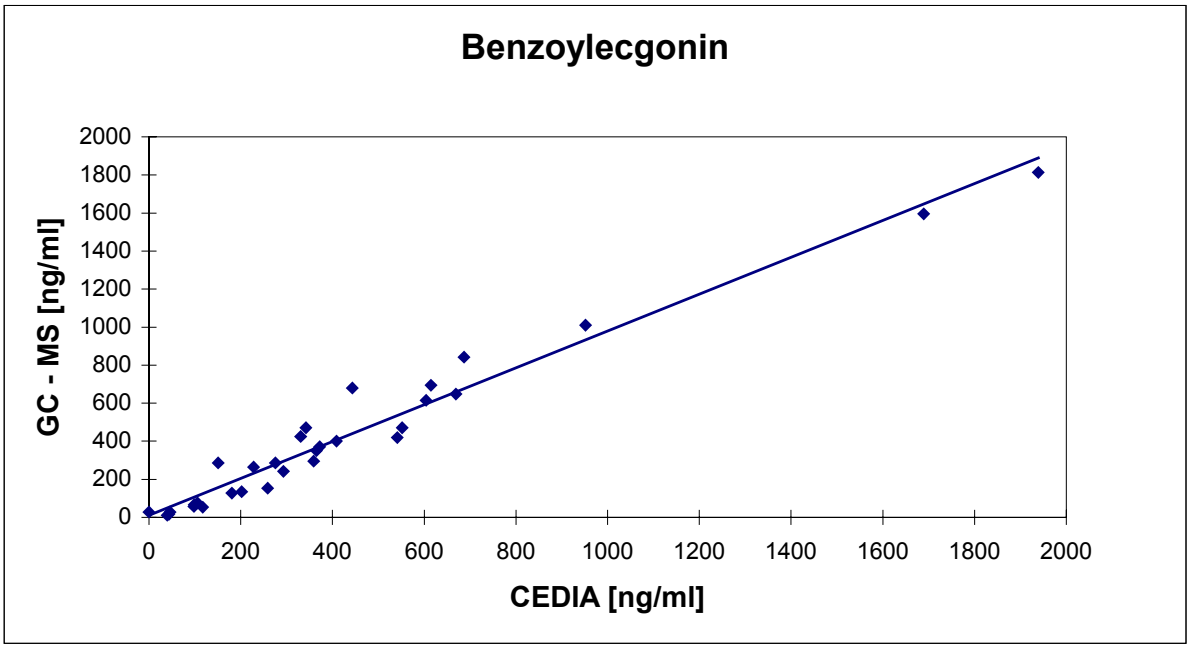

Figure 3: CEDIA BZE Assay in comparison with GC - MS

4.4 Cannabinoids

We measured 113 EDTA-plasma samples positive for the presence of cannabinoids. Between 20 and 65 ng/ml we obtained a mediocre result (see Figure 4). We used a cut-off of 20 ng/ml as shown above. With concentrations higher than 65ng/ml we had an assay saturation and the results of CEDIA had a huge deflection compared with the GC-MS results. Most of the CEDIA results were lower than in the confirmation. Of all the 113 samples we had two false positives, one slightly positive with 27ng/ml and the other with a concentration of 66ng/ml. (Sensitivity 0.98)

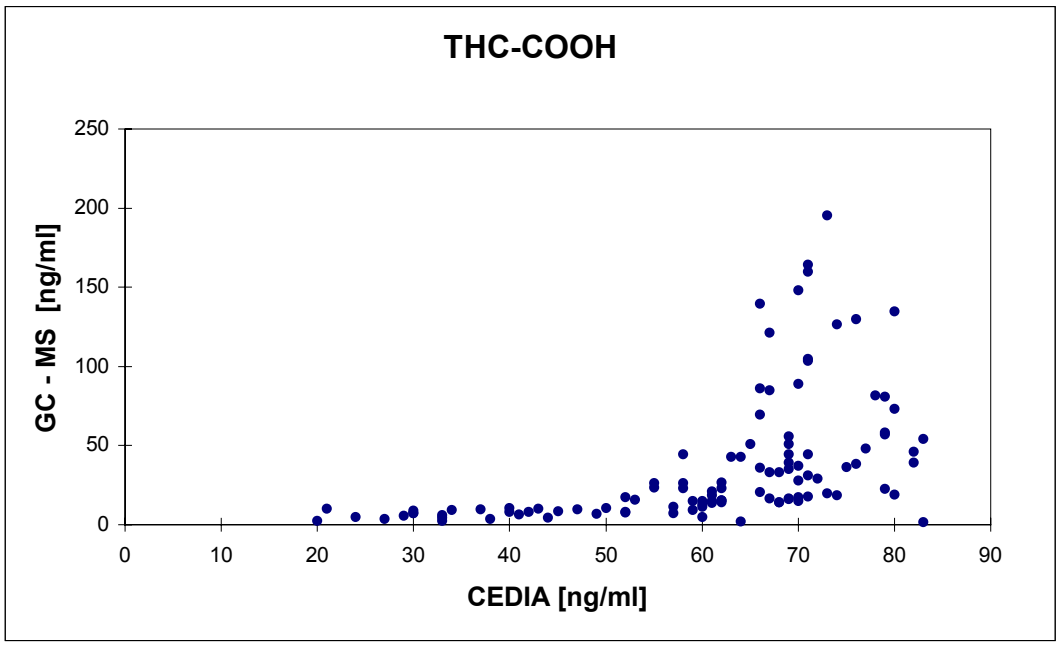

Figure 4: CEDIA THCCOOH – Assay in comparison with GC-MS

4.5 LSD

Within the 250 measured samples we had only five CEDIA® results above the cut – off. Three of them were true – positive, the other two with CEDIA – concentrations of 0.57 and 0.3 ng/ml were negative in the HPLC re-measurement. This may be caused by the interference of the CEDIA® assay with Bromhexine or Ambroxol as shown in urine samples (28).

4.6 Barbiturates

While measuring for the presence of Barbiturates we got three CEDIA® results differing from zero with concentrations of 918, 58 and 21 ng/ml, so they were all three under the cut-off of 1000ng/ml. But in the 918 ng/ml CEDIA sample we found a concentration of 5,810 ng/ml Phenobarbital, so this CEDIA® measurement was false – negative. The two others were true negative.

4.7 Methadone

Within the 250 samples we found 15 samples with CEDIA® - concentrations above the cut – off of 35 ng/ml. One of these results with the CEDIA – results of 185 ng/ml we could not verify with GC – MS, so this is a false – positive result.

4.8 Opiates

92 samples were measured positive for the presence of opiates by CEDIA (see figure 5). All the results could be confirmed by GC – MS so we had no false – positive results.

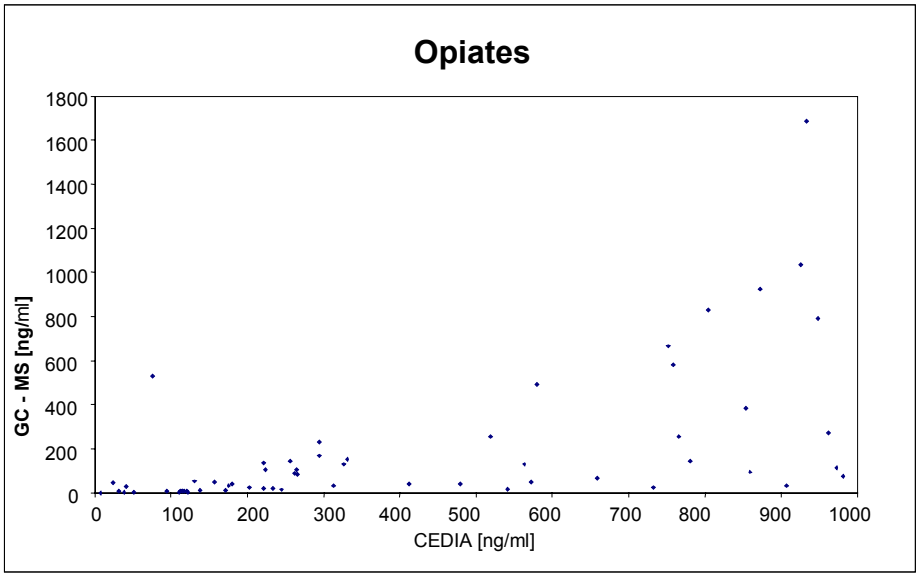

Figure 5: CEDIA® Opiate Assay in comparison with GC – MS

5. Summary

According to the group-specific reactions of the immunoassays, we could only confirm qualitative addictions. Theoretically it would be possible to convert the results with the cross-reactivities into results for other drugs, but this would suppose that there is only one drug present in the sample and no other drug with a cross-reactivity. This was shown for Benzoylecgonin in this study. In most of the cases this would not be possible because of the variety of drugs of abuse and the metabolites. The results printed out are not quantitative measurements, they are substance equivalents per ml e.g. 200ng calibration substance per ml. It is not possible to measure Amphetamines without any pretreatment of the sample if the sample is EDTA - plasma or postmortem blood. The least effort of pretreatment is the dilution 1:1 with water, which gives reliable results for all the measured drug groups.

But we can confirm that the CEDIA DAU – immunoassay is a simple and rapid (less than 5 min (15)) method of blood or plasma screening for drugs of abuse. The small amount of specimen, about 25µl (12) makes it suitable for forensic cases, where often only a small sample is available. Also we did not find interference caused by higher turbidity grades. But perhaps the number of turbid samples (n = 14) was too small. Overall, the CEDIA® DAU is a reliable screening method for the detection of samples where a time consumptive, chromatographic quantitation is appropriate.

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