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## **A rapid screening method for diuretics using automated SPE and LC-MS-MS**

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### **Introduction**

In our laboratory all samples are routinely tested for diuretics. Our normal procedure has used extractive alkylation to simultaneously methylate the diuretics and extract them (Lisi *et al* 1992). The analysis was by GC-MS. This method included 27 diuretics and related compounds and has been successfully used from 1990 to 2002 including the high volume of analyses during the Sydney 2000 Olympic Games (Kazlauskas 2002). Despite the method working successfully for so long there were some reasons to change. These included:

- Some diuretics such as benzthiazide and chlorothiazide do not work well with extractive alkylation.
- Methyl iodide is highly toxic and we desired to reduce its use in the laboratory.
- The existing method was not amenable to further automation.

A number of approaches have recently been published on the improved detection of diuretics ranging from microwave accelerated methylation (Amendola *et al* 2003) to the use of LC-MS with ion trap or triple quadrupole instruments (Sanz-Nebot *et al* 2001, Thieme *et al* 2001, Deventer *et al* 2002). Our new method was required to meet the following criteria:

- No derivatisation was to be required.
- Sample extraction was to be automated.
- All existing compounds including mesocarb and pemoline as well as more diuretics were to be detected.
- All diuretics were to be detected at <100 ng/mL.

- The run time was to be no longer than our existing method (12 minutes).

In order to achieve these objectives it was apparent that the methodology would be based around LC-MS preferably using solid phase extraction (SPE) which could be performed on our existing Gilson ASPEC XL4. The desired detection limit indicated that MS-MS would be needed and the short run time indicated the need for a relatively short LC column with a fast gradient. There are two means of achieving MS-MS either in space or in time (tandem quadrupole or ion trap). Two LC-MS-MS instruments were evaluated a Micromass Quattro Micro and an Agilent LC-MSD-Trap. Samples run on these instruments indicated a marked sensitivity advantage with the tandem quadrupole in MRM mode.

## **Experimental**

All reagents were of AR or HPLC grade. Water was from a Milli-Q water purification system. The diuretics were from the National Analytical Reference Laboratory (NARL) or from the manufacturers.

The sample extraction procedure was:

- To each 2 mL of urine was added 2 mL of pH 5 acetate buffer and 100  $\mu$ L of mefruside internal standard (10  $\mu$ g/mL). The tubes were vortexed and the analytes extracted by passage of the sample through a Varan ABS ELUT Nexus SPE column (60 mg, 3 mL), followed by a 1 mL water wash, a 1 mL wash with 20% methanol in water, and elution with 2 mL of methanol.
- The methanol extract was evaporated to dryness under a stream of nitrogen and reconstituted in 200  $\mu$ L of 50% methanol in water.

The HPLC separation was carried out using a Waters 2795 Alliance separation module with a C18 column (Lettish Prevail, 50 mm  $\times$  2.1 mm  $\times$  3  $\mu$ m) protected by a C18 guard column. The following ternary mobile phase gradient was formed by solvent A (2% aqueous formic acid), solvent B (water), and solvent C (acetonitrile) at a flow rate of 0.2 mL/Min: constant 10% A, 0% C (0 to 1 Min), 0 to 80% C (1 to 6.5 Min), 80% C (6.5 to 7.5 Min), 80 to 0% C (7.5 to 8 Min), and 0% C (8 to 11 Min). The MS-MS detection was carried out using a Micromass Quattro Micro with a Z spray API interface. The spray conditions of the interface were: desolvation

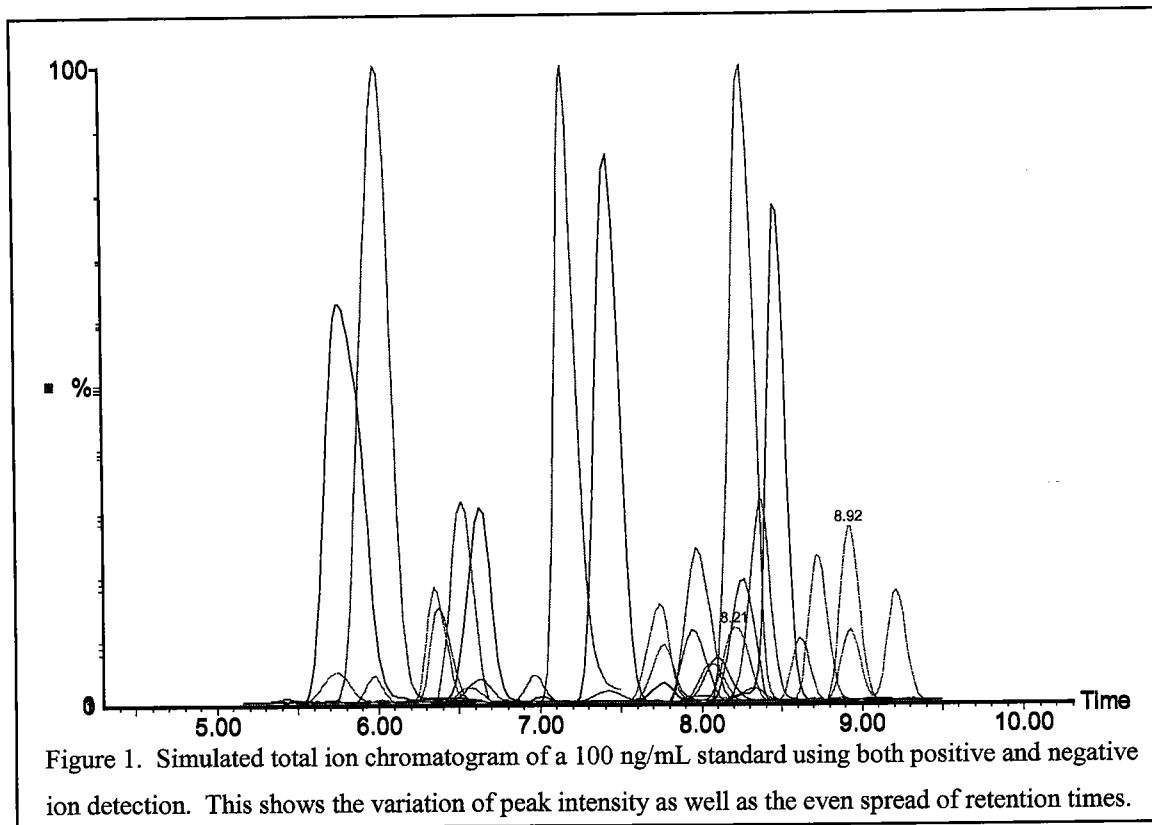
temperature 180°C, desolvation gas flow 550 L/hr, and cone gas flow 50 L/hr. The capillary was set at 3.5 kV and the substance specific cone voltage and collision energy were optimised for each compound. Argon was used as the collision gas at a pressure of  $3.3 \times 10^{-3}$  mbar. Multiple reaction monitoring (MRM) was carried out at the optimum settings using both positive and negative ion modes. Six MRM functions were used:

- 6 channels ES- 5.15 to 6.65 mins
- 6 channels ES+ 5.50 to 7.50 mins
- 4 channels ES- 6.35 to 7.75 mins
- 7 channels ES+ 7.20 to 8.35 mins
- 8 channels ES- 7.50 to 9.50 mins
- 6 channels ES+ 7.85 to 9.50 mins

## Results and Discussion

The method was an extension of the LC-MS-MS procedure we had developed in 2000 for a restricted range of diuretics which used ethyl acetate for extraction and detected positive ions only (Fouracre and Kazlauskas 2001). For the initial evaluation of the best conditions for MS detection the ethyl acetate extraction procedure described in this method was used. Seven out of the 36 compounds to be analysed could not be detected in positive ion mode. These were amiloride, canrenone, clorexolone, metolazone, pemoline, spironolactone and triamterene. For the best sensitivity it was found that 22 compounds should be analysed in positive ion mode and 14 compounds in negative ion mode. The simulated TIC found for both modes of ion detection from a 100ng/mL standard is shown in Figure 1. The great variation in peak heights is an indication of the widely varying responses observed for the different analytes. During this development stage it was observed that for some urine samples the compounds althiazide, benzthiazide, and polythiazide could not be recovered. These compounds all have a sulfide side chain and their oxidation to sulfoxides and sulfones in the presence of ethyl acetate has been reported (Thieme et al 2001). Further work confirmed that oxidation had occurred but also found that the oxidation only occurred in some 20% of the urines tested. The occurrence of the oxidation did not appear to be related solely to the purity of the ethyl acetate as several urines repeatedly and

reproducibly underwent the oxidation whilst most other urines did not. This result meant that ethyl acetate could not be used routinely to extract these three diuretics and provided an additional reason to change to solid phase extraction.



Figures 2 and 3 show two pages from a three page printout that is generated for each sample. Figure 2 is from a urine spiked with 31 analytes at a concentration of 100 ng/mL whilst Figure 3 is from a typical urine. All 31 analytes are readily detected at 100 ng/mL both graphically and in the text results summary. In the typical urine no peaks are detected and there are no text results (see Figure 3 where the area and height values – columns 5 & 6 – are negligible other than for the internal standard). This is the normal result for the vast majority of urine samples tested so far (more than 3000). The recoveries of the drugs excreted as parent compounds were determined by spiking seven replicates of a blank urine with each analyte at a concentration of 50ng/mL, and comparing these results with an unextracted standard. A summary of the results obtained are set out in Table 1.

The robustness of the method was evaluated by taking urine samples from 40 individuals and spiking each with all the parent compounds at a concentration of 50ng/mL. The results showed that all analytes could be detected in all 40 urine

samples, however for some samples the recoveries of dichlorphenamide and/or quinethazone were lower than expected although the peaks were still clearly observable. It was noted that there was a correlation between high urine specific gravity and low recovery of dichlorphenamide and/or quinethazone.

Acetazolamide	Althiazide	Amiloride
Bendroflumethiazide	Benzthiazide	Bumetanide
Canrenone	Clorexolone	Chlorothiazide
Chlorthalidone	Clopamide	Cyclopenthiiazide
Cyclothiazide	Dichlorophenamide	Ethacrynic Acid
Furosemide	Hydrochlorothiazide	Hydrofluthiazide
Indapamide	Methazolamide	Metolazone
Piretanide	Polythiazide	Quinethazone
Spironolactone	Torasemide	Triamterene
Trichlormethiazide	Xipamide	Pemoline
Probenecid		

	Mean	Minimum	Maximum
% Recovery	101	65	125
SD	14	7	26

Table 1. Summary of recovery data and the 31 analytes used.

An estimate of the level of detection was made by spiking urines with the drugs excreted as parent compounds. It was found that of the 31 compounds only althiazide could not be routinely detected at 20 ng/mL or below. Table 2 illustrates the results obtained when a urine spiked with 1 ng/mL of the drugs was analysed with 17 compounds being detected with a signal to noise ratio of 3 or more.

Diuretic	S/N	Diuretic	S/N
Amiloride	10	Hydrofluthiazide	2
Benzthiazide	3	Indapamide	>10
Bumetanide	10	Metolazone	10
Canrenone	6	Piretanide	>10
Clorexolone	>10	Polythiazide	2
Chlorothiazide	2	Spironolactone	>10
Clopamide	>30	Torasemide	>30
Cyclopenthiiazide	6	Triamterene	>30
Dichlorophenamide	3	Xipamide	7
Ethacrynic acid	6	Pemoline	>20

Table 2. Signal to noise ratios obtained from a urine sample spiked at 1 ng/mL.

The method has been in routine use for several months and has demonstrated its reliability and robustness. The only instrumental maintenance required has been the routine replacement of guard columns and washing of the cone of the electrospray. Figure 4 shows a real sample from an athlete who had taken a combined diuretic containing amiloride and chlorothiazide. The top row shows the relevant ion traces for this sample clearly showing the presence of amiloride, chlorothiazide and its metabolite hydrochlorothiazide. The presence of these compounds was confirmed using our GC-MS extractive alkylation procedure and the sample declared positive. The middle row shows a typical blank sample. The bottom row is from another sample that has smaller peaks in the amiloride, chlorothiazide, and hydrochlorothiazide windows. The concentrations were low being below 20 ng/mL and it was not possible to confirm their presence using GC-MS despite a larger urine sample being taken for analysis. Because the urine specific gravity was not low no further action was taken and the sample was declared negative. Subsequent events revealed that both samples had been taken from the same subject but at different dates with the suspect sample being taken very late in the excretion of the drugs.

## **Conclusions**

The current method analyses 29 diuretics as their parent compounds, the oxo-metabolite of mefruside, pemoline, probenecid, mesocarb hydroxy sulfate, chloroamidophenamide and triflumethylamidophenamide (hydrolysis of benzothiadiazines). The Gilson ASPEC XL4 used for the extraction processes 16 samples per hour, whilst the LC-MS-MS can analyse 5 samples per hour. The sensitivity achieved is more than adequate to achieve the 100 ng/mL minimum required proficiency limit proposed in the WADA international standard for laboratories (WADA 2002). In fact in order to ensure standardisation with the IOC/WADA accredited laboratories the confirmation is performed using the GCMS procedure which meets the MRPL required but is considerably less sensitive than the LCMS procedure used for screening. The method has been in routine use in our laboratory for over six months and has replaced our previous GC-MS screen.

## **References**

A.M Lisi, R Kazlauskas, and G.J. Trout , J. Chromatogr. B, Biomed. Appl. 581 57-63 (1992).

R. Kazlauskas, Clin. Biochemist Reviews, 23 35-51 (2002).

L. Amendola, C. Colamonici, M. Mazzarino, F. Botre, Anal. Chim. Acta, 475 125-136 (2003).

V. Sanz-Nebot, I. Toro, R. Berges, R. Ventura, J. Segura and J. Barbosa, J Mass Spectrom., 36 652-657 (2001).

D. Thieme, J. Grosse, R. Lang, R.K. Mueller and A. Wahl, J. Chromatogr. B, 757 49-57 (2001).

K. Deventer, F.T. Delbeke, K. Roels, P. Van Eenoo, Biomed. Chromatogr., 16 529-535 (2002).

C. Fouracre and R. Kazlauskas in: W. Schänzer, H. Geyer, A. Gotzmann U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis (9), Sport und Buch Strauß, Köln, p321-326 2001.

Minimum required performance limits for diuretics in the proposed WADA approved international standard for laboratories version 3.

[http://www.wada-ama.org/docs/web/standards\\_harmonization/code/min\\_limits\\_1\\_2.pdf](http://www.wada-ama.org/docs/web/standards_harmonization/code/min_limits_1_2.pdf) accessed 9<sup>th</sup> July 2002.



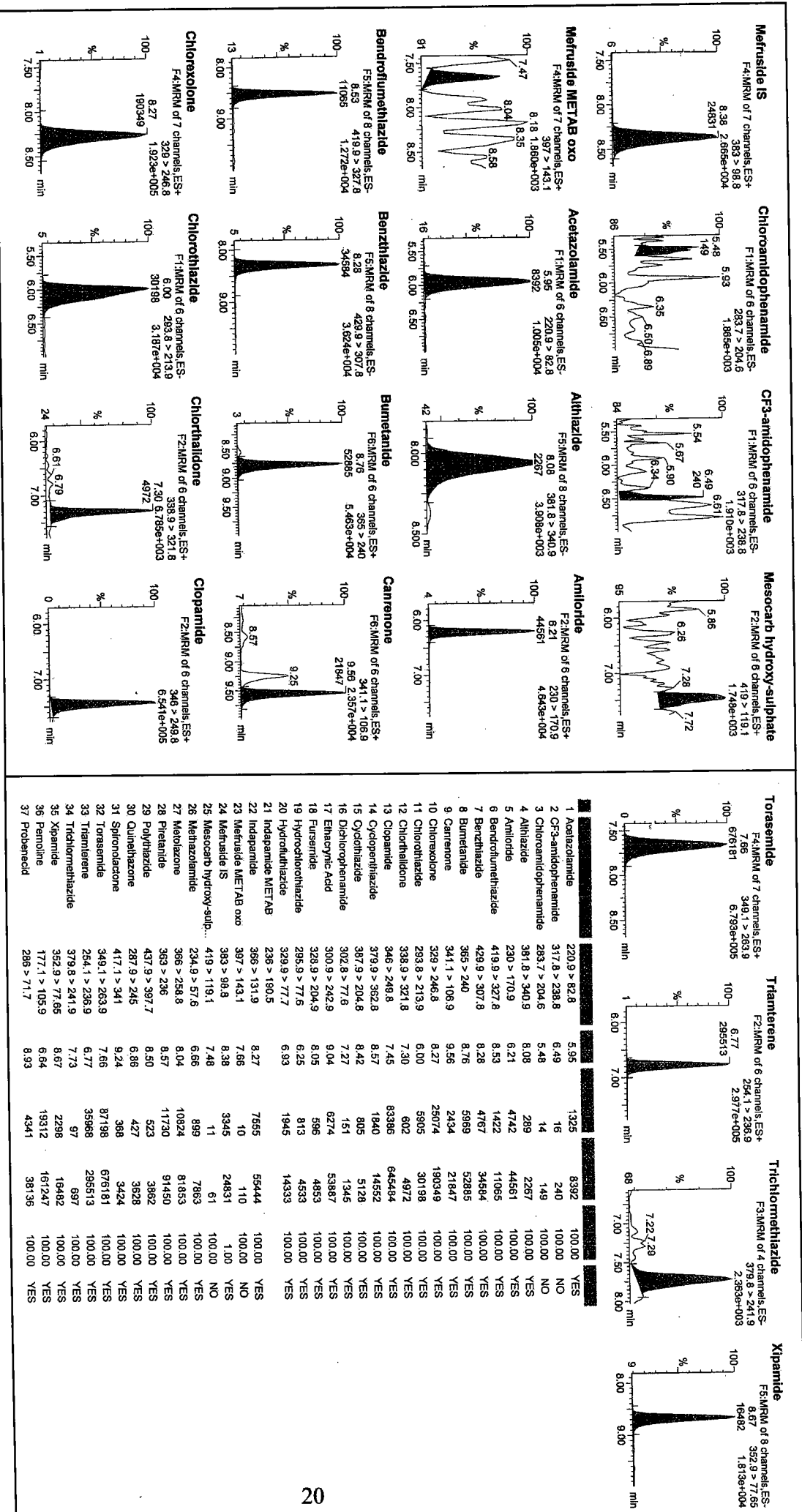


Figure 2. Pages 1 and 3 of a 3 page printout for a urine spiked at 100ng/ml. The urine is not spiked with hydroxymesocarb sulphate, mefruside metabolite or the hydrolysis products of the benzothiazides

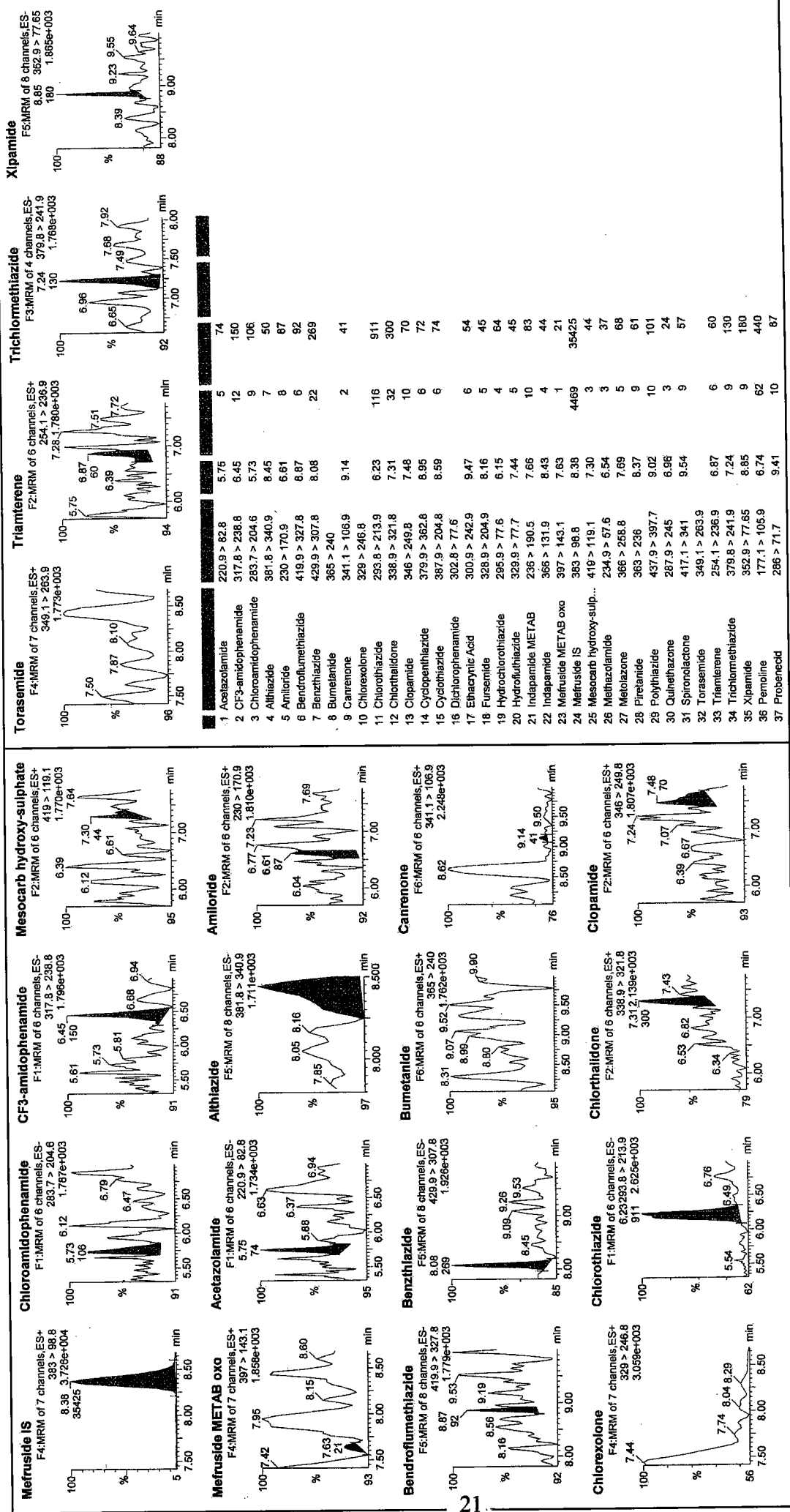


Figure 3. Pages 1 and 3 of a 3 page printout from a typical urine sample.

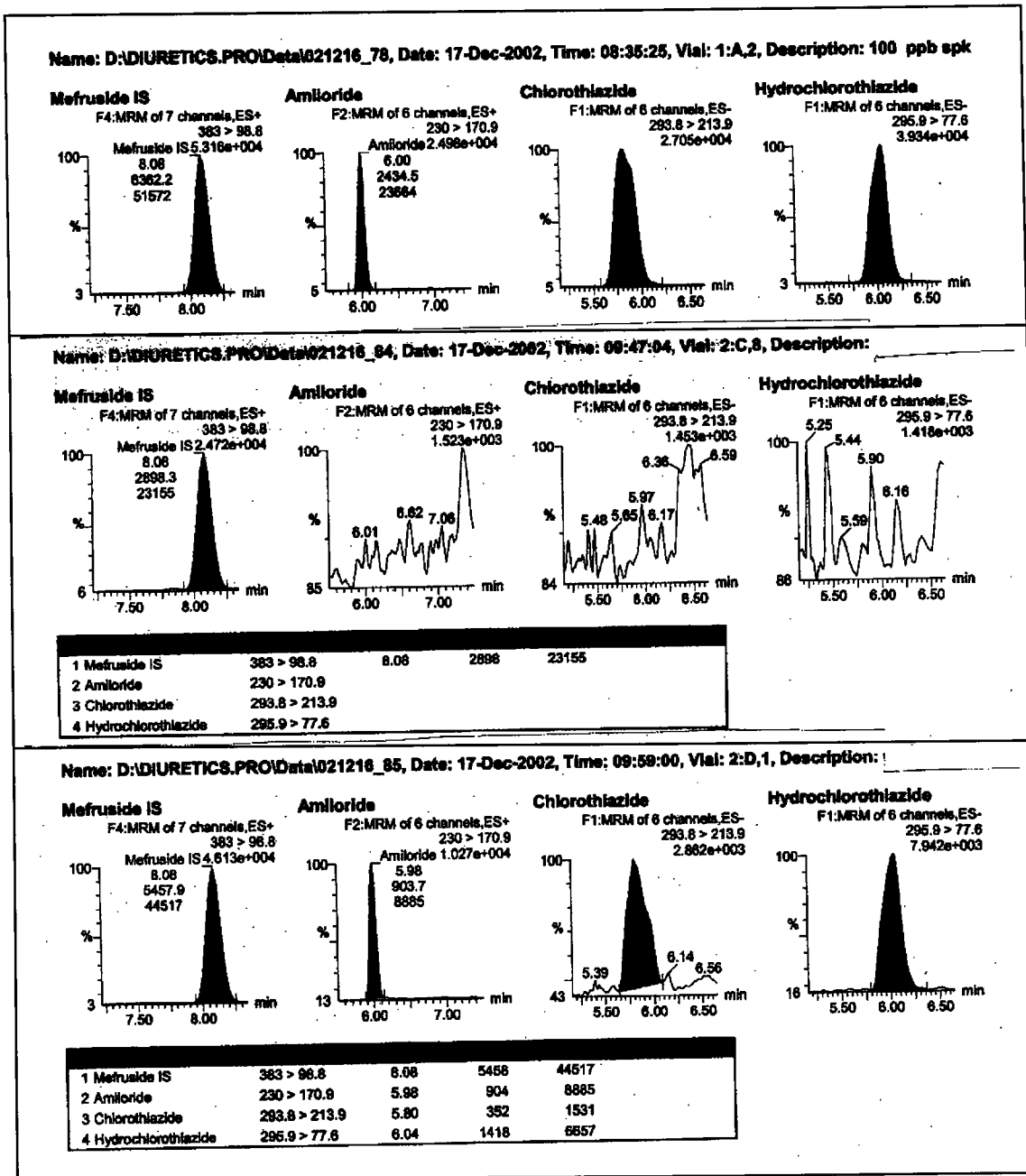


Figure 4 Screening results obtained from a positive urine sample (top row), a blank urine (middle row), and a suspect urine positive (bottom row).