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THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF MAJOR METAMIZOLE METABOLITES IN SERUM AND URINE

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SUMMARY

Thin-layer chromatographic (TLC) methods were developed for pharmacokinetic studies of major metamizole metabolites in serum and urine. These methods proved practicable, selective, accurate and sensitive with detection limits as follows: 4-methylaminoantipyrine 0.6 μg/ml serum and 2.0 μg/ml urine; 4-aminoantipyrine 0.16 μg/ml serum and 1.0 μg/ml urine; 4-acetylaminoantipyrine 0.14 μg/ml serum and 0.6 μg/ml urine; 4-formylaminoantipyrine 0.12 μg/ml serum and 1.0 μg/ml urine.

Samples of 250 μl suffice for TLC analysis. Following chromatographic separation, detection is performed in the ultraviolet range at 265 nm. Serum and urine levels were determined following a single oral dose of 1 g of metamizole sodium to eight volunteers. These results were compared with those following an accidental overdose of 49 g.

INTRODUCTION

Metamizole (Fig. 1) is an effective and widely used analgesic drug. The drug and its fate within the body have been characterized mainly by using radiolabelled material. Metamizole in solution rapidly undergoes hydrolysis [1] to yield 4-methylaminoantipyrine, which in man [2] is further metabolized to 4-aminoantipyrine, 4-acetylaminoantipyrine and 4-formylaminoantipyrine. Known metabolites account for approx. 77% of total radioactivity in serum

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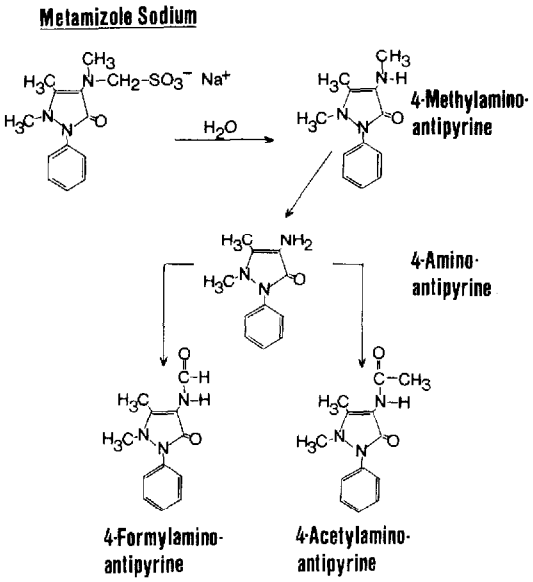

Fig. 1. Structure of metamizole sodium and metabolites.

(2—8 h post administration) and approx. 66% of total radioactivity in urine (0—24 h).

Biopharmaceutical and pharmacokinetic data in rat, dog and man were reported by Christ et al. [3]. Single oral doses of 480 mg of [^{14}C]metamizole administered to eleven human volunteers were rapidly and almost completely absorbed. The serum half-life was calculated from total radioactivity data as 7 ± 1 h up to 8 h post administration. The urinary half-life was 10 ± 1 h up to 4 days post administration. During that period, 90 ± 5% of the dose was renally excreted.

Further, it would be valuable to characterize the pharmacokinetics of commercial, i.e. non-labelled, preparations of the drug. Since for such purposes, large numbers of individual blood samples are usually required, it would be desirable to use small serum aliquots per analysis.

The present report describes selective thin-layer chromatographic (TLC) assays for 4-methylaminoantipyrine, 4-aminoantipyrine, 4-acetylaminoantipyrine and 4-formylaminoantipyrine in serum and urine. In one single extraction step, 250-μl samples are cleaned-up for chromatographic analysis.

EXPERIMENTAL

Reagents

The reagents used were buffer pH 10 AR, trichloroacetic acid AR (100 g/l), dichloromethane AR, diethyl ether AR, chloroform AR (freshly distilled), methanol AR and concentrated ammonia solution (25%) AR. The solvent system was chloroform—methanol—diethyl ether—concentrated ammonia (25:5:6:1).

Equipment

A Zeiss KM3 chromatogram spectrophotometer with microoptics and a Servogor^{R} 210 (Metrawatt) recorder were used. Separation was performed on silica gel HPTLC plates F 254* (No. 5642, E. Merck, Darmstadt, G.F.R.) in a Camag twin-trough HPTLC chamber 20 cm × 10 cm (No. 25254). For sample clean up and spotting, a Vortex^{R} mixer, a centrifuge, glass-stoppered tubes (approx. 8 ml), conical glass-stoppered tubes (approx. 8 ml) and a Desaga Autospotter^{R}** were used.

Sample preparation

Serum. In a glass-stoppered tube, 250 µl of serum were deproteinized with 50 µl of trichloroacetic acid solution. Following centrifugation (15 min), 100 µl of deproteinized serum were transferred to a second tube and treated with 1 ml of buffer pH 10. The mixture was extracted with 5 ml of dichloromethane for 30 sec on a Vortex mixer. The phases were separated by centrifugation and 4 ml of the organic phase were transferred to a conical tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 100 µl of chloroform. Using the Autospotter, 70 µl were transferred onto the HPTLC plate as a series of small consecutive droplets, approx. 100 nl each.

Urine. In a glass-stoppered tube, 250 µl*** of urine were treated with 1 ml of buffer pH 10. The urine was extracted with 5 ml of chloroform for 30 sec on the Vortex mixer. The phases were separated by centrifugation (5 min) and 70 µl were transferred onto the HPTLC plate.

Chromatography

The twin-trough HPTLC developing chamber contained 20 ml of solvent in one compartment. The plate was developed in the dark without previous saturation over a distance of 7 cm. R_F values were: 4-methylaminoantipyrine 0.80, 4-aminoantipyrine 0.65, 4-acetylaminoantipyrine 0.50, and 4-formylaminoantipyrine 0.40.

Measurements were carried out in the direction of the solvent flow with an effective slit (microoptics) of 4.5 mm × 0.15 mm at a wavelength of 265 nm (Fig. 2), scanning speed 50 mm/min and paper speed 150 mm/min. Peak heights were evaluated and quantified by means of a calibration graph based on parallel analyses of standards on the same plate (Figs. 3 and 4).

RESULTS

Serum

The compounds were admixed to blank serum in five concentrations over the analytical ranges indicated in Table I. Each admixture was split into six

*As an advantage, the fluorescence indicator allows a fast overview of the chromatographic result before scanning. The baseline quality, as we experienced, is not markedly affected when extracts from biological samples are used.

**Modified version, Tygon^{R} tubes of larger diameter (Technicon, flow-rated, code 116-0549-09, white) and 60-cm long Hostaflon^{R} tubes were used.

***Samples of 100 µl were used for concentrations greater than 50 µg/ml urine.

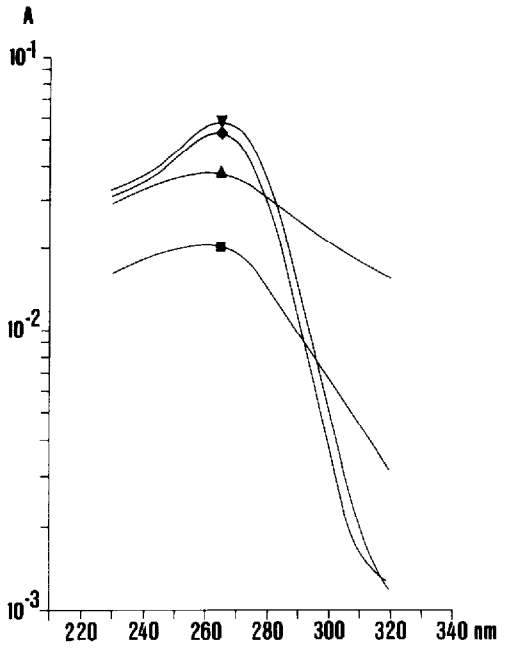

Fig. 2. In situ ultraviolet spectra on an HPTLC plate, 70 ng/spot: (■), 4-methylaminoantipyrine; (▲), 4-aminoantipyrine; (♦), 4-acetylaminoantipyrine; (▼), 4-formylaminoantipyrine.

TABLE I

DETERMINATION OF METAMIZOLE METABOLITES IN SERUM BY TLC: RECOVERY AND ASSAY PRECISION

n = 6 determinations, concentration in μg/ml.

4-Methylaminoantipyrine		4-Aminoantipyrine		4-Acetylaminoantipyrine		4-Formylaminoantipyrine	
Added	Found	Added	Found	Added	Found	Added	Found
20.0	19.9 ± 0.2	5.00	5.1 ± 0.06	5.0	5.0 ± 0.06	5.0	5.0 ± 0.05
10.0	10.3 ± 0.4	2.0	1.9 ± 0.11	2.0	2.0 ± 0.08	2.0	2.1 ± 0.08
5.0	5.1 ± 0.3	1.00	0.95 ± 0.07	1.00	0.97 ± 0.11	1.00	1.07 ± 0.10
2.0	2.1 ± 0.3	0.50	0.53 ± 0.08	0.50	0.52 ± 0.04	0.50	0.51 ± 0.05
1.0	0.7 ± 0.2	0.25	0.26 ± 0.08	0.25	0.24 ± 0.03	0.25	0.22 ± 0.04
Blank	0	Blank	0	Blank	0	Blank	0

portions of 250 μl, so that six equal series were formed. Each series was then analyzed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria of an analytical method are selectivity, accuracy, precision and sensitivity. The corresponding parameters were derived from the analytical results given in Table I. As regards selectivity, the assay is free from interferences for all substances (Fig. 3). Accuracy was considered to be the deviation (bias) of the mean value of the results from the amount added. In the case of all substances, the average accuracy was < 30 ng/ml. Regression coefficients were greater than 0.999. Assay precision was defined in terms of the standard deviation (S.D.), which was constant in the concentration range considered. Sensitivity was expressed by the detection limit (D.L.) and was taken

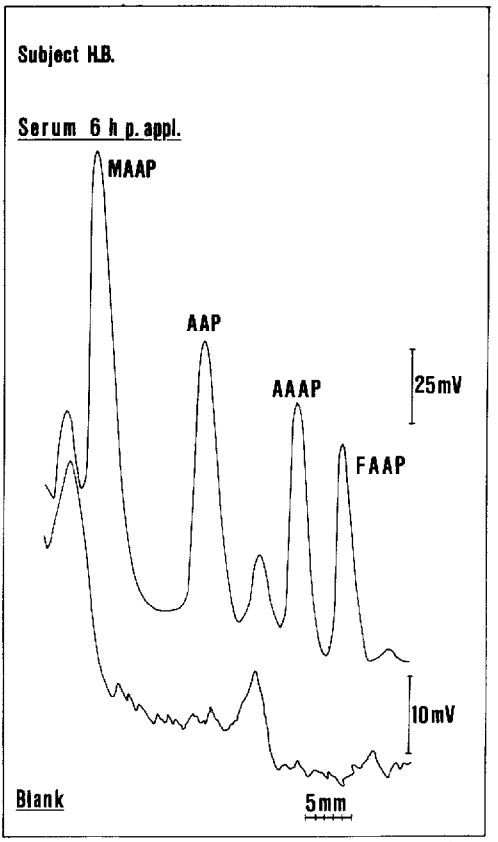

Fig. 3. Determination of serum 4-methylaminoantipyrine (MAAP) (3.2 μg/ml), 4-aminoantipyrine (AAP) (1.2 μg/ml), 4-acetylaminoantipyrine (AAAP) (1.2 μg/ml) and 4-formylaminoantipyrine (FAAP) (1.0 μg/ml) compared to a blank.

as precision × 2. Thus, for
4-methylaminoantipyrine, precision = 0.3 ± 0.1 μg/ml, D.L. = 0.6 μg/ml;
4-aminoantipyrine, precision = 0.08 ± 0.02 μg/ml, D.L. = 0.16 μg/ml;
4-acetylaminoantipyrine, precision = 0.07 ± 0.04 μg/ml, D.L. = 0.14 μg/ml;
4-formylaminoantipyrine, precision = 0.06 ± 0.03 μg/ml; D.L. = 0.12 μg/ml.

Urine

The compounds were admixed to blank urine in concentrations over the range 1—50 μl/ml urine. Each admixture was split into six portions of 250 μl, so that six equal series were formed. Each series was then analyzed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria of the method for urine were defined by the corresponding parameters abstracted from the analytical results given in Table II. As regards selectivity, the assay is free from interferences for all substances (Fig. 4). In the case of all substances, the average accuracy was < 0.2 μg/ml. Regression coefficients were greater than 0.999. Precision as defined in terms of the standard deviation (S.D.) was constant in the concentration range considered. Sensitivity was expressed by the detection limit (D.L.), i.e. precision × 2.

TABLE II

DETERMINATION OF METAMIZOLE METABOLITES IN URINE BY TLC: RECOVERY AND ASSAY PRECISION

n = 6 determinations, concentration in μg/ml.

4-Methylaminoantipyrine		4-Aminoantipyrine		4-Acetylaminoantipyrine		4-Formylaminoantipyrine	
Added	Found (mean ± S.D.)	Added	Found (mean ± S.D.)	Added	Found (mean ± S.D.)	Added	Found (mean ± S.D.)
50.0	50.3 ± 0.7	50.0	49.7 ± 0.5	50.0	49.5 ± 0.3	50.0	49.2 ± 0.3
20.0	19.6 ± 2.0	20.0	20.5 ± 1.1	20.0	20.0 ± 0.5	20.0	20.7 ± 1.3
10.0	9.3 ± 0.5	10.0	9.4 ± 0.6	10.0	10.4 ± 0.5	10.0	10.3 ± 0.5
5.0	5.4 ± 0.8	5.0	4.7 ± 0.5	5.0	5.3 ± 0.3	5.0	5.2 ± 0.6
2.0	2.2 ± 0.9	2.0	1.9 ± 0.2	2.0	2.0 ± 0.3	2.0	2.1 ± 0.4
1.0	0	1.0	1.1 ± 0.2	1.0	0.8 ± 0.2	1.0	0.8 ± 0.2
Blank	0	Blank	0	Blank	0	Blank	0

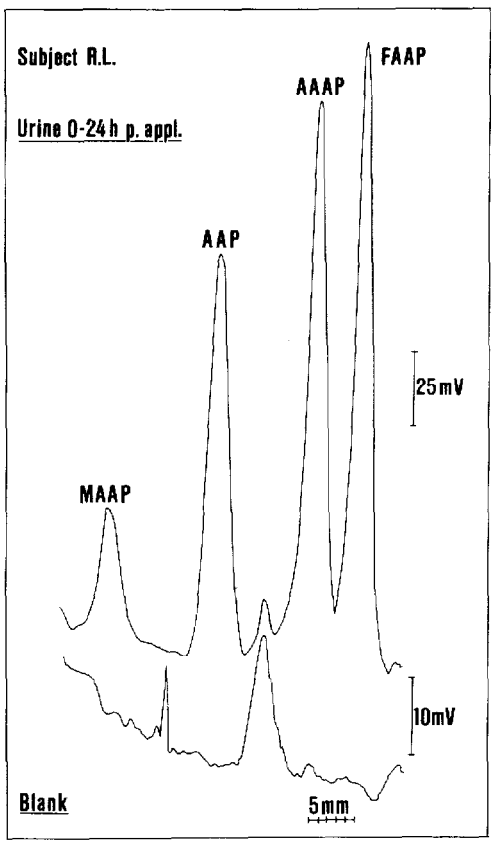

Fig. 4. Determination of urinary 4-methylaminoantipyrine (MAAP) (6.8 μg/ml), 4-aminoantipyrine (AAP) (23 μg/ml), 4-acetylaminoantipyrine (AAAP) (53 μg/ml) and 4-formylaminoantipyrine (FAAP) (58 μg/ml) compared to a blank.

Thus for

4-methylaminoantipyrine, precision = 1.0 ± 0.6 μg/ml, D.L. = 2.0 μg/ml;
4-aminoantipyrine, precision = 0.5 ± 0.3 μg/ml, D.L. = 1.0 μg/ml;
4-acetylaminoantipyrine, precision = 0.3 ± 0.1 μg/ml, D.L. = 0.6 μg/ml;
4-formylaminoantipyrine, precision = 0.5 ± 0.4 μg/ml, D.L. = 1.0 μg/ml.

Pharmacokinetics

Metamizole sodium was administered as a single oral dose of 1 g to eight human volunteers*. This dosage corresponds to that generally recommended in therapeutic use. Serum samples were obtained before and at 2, 6 and 24 h following treatment; urine was collected for 24 h.

Mean serum and urine concentrations are presented in Tables III and IV, respectively. The overall serum concentration up to 6 h post administration was dominated by levels of methylaminoantipyrine, later by those of acetyl- and formylaminoantipyrine.

TABLE III

MEAN SERUM CONCENTRATIONS FOLLOWING A SINGLE ORAL DOSE OF 1 g OF METAMIZOLE SODIUM

n = 8 volunteers, concentration in μg/ml, mean ± S.D.

h post dose	4-Methylamino-antipyrine	4-Amino-antipyrine	4-Acetylamino-antipyrine	4-Formylamino-antipyrine	Overall concentration (drug)
0	0	0	0	0	0
2	9 ± 2	1.0 ± 0.3	0.8 ± 0.6	1.0 ± 0.5	19 ± 3
6	5 ± 1	1.4 ± 0.8	1.8 ± 1.0	1.7 ± 0.7	15 ± 2
24	0.1 ± 0.1	0.2 ± 0.6	1.5 ± 0.6	0.8 ± 0.3	4 ± 1

TABLE IV

MEAN URINARY CONCENTRATIONS FOLLOWING A SINGLE ORAL DOSE OF 1 g OF METAMIZOLE SODIUM, 0—24 h COLLECTION PERIOD

n = 8 volunteers, concentration in μg/ml, mean ± S.D.

4-Methylamino-antipyrine	4-Aminoantipyrine	4-Acetylamino-antipyrine	4-Formylamino-antipyrine
7 ± 3	18 ± 15	95 ± 55	51 ± 19

From serum data, metabolite half-lives of approx. 3 h for 4-methylamino-antipyrine and approx. 6 h for 4-aminoantipyrine were calculated. Half-lives of 4-acetyl- and 4-formylaminoantipyrine were estimated as approx. 10—15 h (Fig. 5). An overall half-life of approx. 10 h was in accordance with ^{14}C-data from Christ et al. [3].

*This study was performed by Drs. W. Rupp and M.J. Badian, Hoechst AG.

Mean areas under the serum curves up to 24 h post administration are given in Table V.

Within 24 h post administration, 35 ± 4% of the dose was accounted for in urine as the sum of excreted metabolites, predominantly acetyl- and formyl-

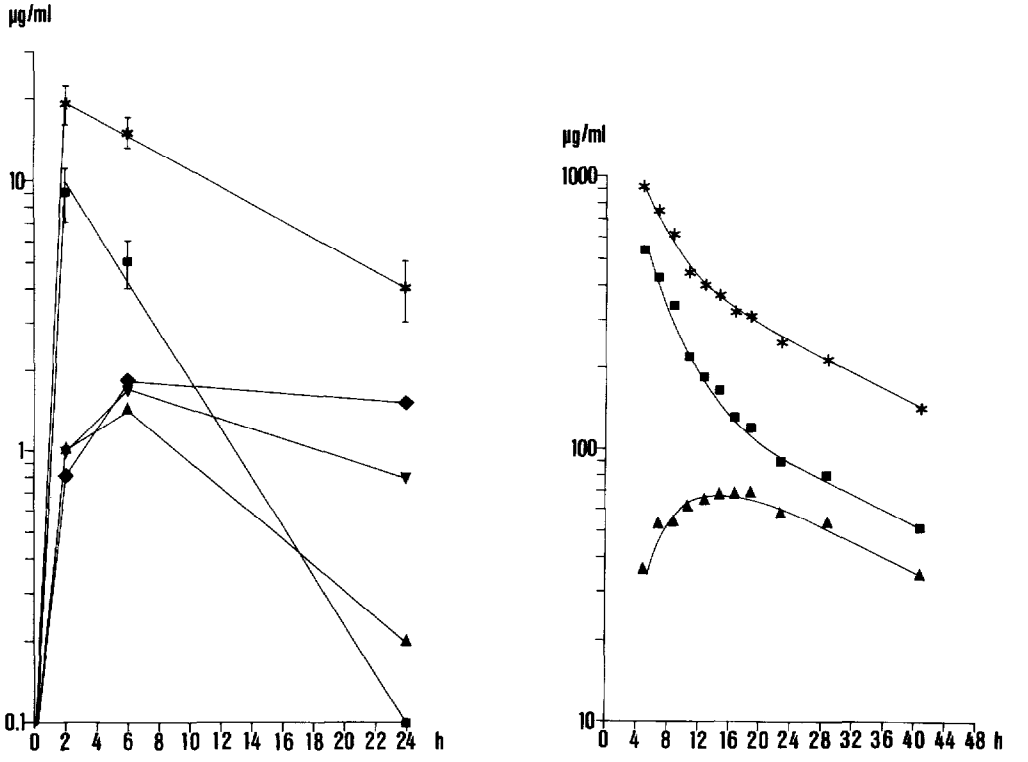

Fig. 5. Serum pharmacokinetics following a single oral dose of 1 g of metamizole sodium, mean serum levels of eight volunteers. (*), Molar sum expressed as metamizole sodium; (■), 4-methylaminoantipyrine; (▲), 4-aminoantipyrine; (◆), 4-acetylaminoantipyrine; (▼), 4-formylaminoantipyrine.

Fig. 6. Serum pharmacokinetics following an accidental overdose of 49 g of metamizole sodium. (*), Molar sum expressed as metamizole sodium; (■), 4-methylaminoantipyrine; (▲), 4-aminoantipyrine.

TABLE V

MEAN AREAS UNDER SERUM CURVES (AUC) 0—24 h, 1 g DOSE

n = 8 volunteers.

	AUC (µg h ml^{-1}) (mean ± S.D.)
4-Methylaminoantipyrine	85 ± 23
4-Aminoantipyrine	20 ± 11
4-Acetylaminoantipyrine	36 ± 17
4-Formylaminoantipyrine	29 ± 11
Overall metamizole sodium	255 ± 34

aminoantipyrine (Table VI). These findings correspond with data from Volz and Kellner [2].

Further, the analytical methods were applied in the case of attempted suicide by an 18-year-old girl. The extreme overdose of 49 g of metamizole sodium was well tolerated [4]. In contrast to the 1 g oral dosage, overall concentrations in serum and urine were dominated by methylaminoantipyrine and aminoantipyrine levels. The overall serum concentration of approx. 1000 μg/ml 5 h post administration decreased in two phases with half-lives of 2.5 h and 20 h (Fig. 6). Methylaminoantipyrine serum levels of 540 μg/ml 5 h post administration decreased with half-lives of 2.5 h and approx. 20 h reaching approx. 50 μg/ml after 48 h.

TABLE VI

MEAN CUMULATIVE URINARY EXCRETION 0—24 h, 1 g DOSE

n = 8 volunteers; mean ± S.D.

	Amount excreted (mg)	Percentage of dose
4-Methylaminoantipyrine	10 ± 4	1.5 ± 0.6
4-Aminoantipyrine	27 ± 23	4.4 ± 3.8
4-Acetylaminoantipyrine	135 ± 60	18.3 ± 8.2
4-Formylaminoantipyrine	76 ± 24	11.0 ± 3.5
Overall excretion as metamizole sodium	354 ± 44	35 ± 4

TABLE VII

CUMULATIVE URINARY EXCRETION 0—24 h, 49 g OVERDOSE

1 patient.

	Approx. amount excreted (g)	Percentage of dose
4-Methylaminoantipyrine	2.7	8.8
4-Aminoantipyrine	2.5	8.8
4-Acetylaminoantipyrine	0.9	2.7
4-Formylaminoantipyrine	0.7	2.2
Overall excretion as metamizole sodium	11	22

Maximum aminoantipyrine serum levels of 68 μg/ml were observed between 15 and 19 h post administration. They declined by the same terminal half-life of approx. 20 h, reaching approx. 35 μg/ml after 48 h. Acetylaminoantipyrine

and formylaminoantipyrine serum levels were below 6 μg/ml. Within 24 h post administration, approx. 22% of the dose was accounted for in urine as the sum of excreted metabolites (Table VII).

The overall urinary excretion up to 24 h may be assumed to contain at least the same percentage of dose absorbed as after the 1 g dose. Therefore in the case of the 49 g overdose, an absorbed fraction of at least 30 g of metamizole sodium may be assumed.

CONCLUSION

Metamizole assay in serum and urine was carried out to demonstrate quantitative TLC as a practicable, selective and reliable analytical tool adequate for drug/metabolite research in the therapeutic range and for handling samples from acute overdosing, where unexpected effects may occur and fast results are needed.

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