Journal of Chromatography, 415 (1987) 35–44
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3488

DETERMINATION OF METHYLPREDNISOLONE IN RAT TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

MAJVI JONUNG, SVEN BJÖRKMAN* and JAN IDVALL

Department of Anaesthesiology and Hospital Pharmacy, Malmö General Hospital, S-21401 Malmö (Sweden)

(Received September 22nd, 1986)

SUMMARY

Methylprednisolone was determined in various types of rat tissue following an intravenous injection of methylprednisolone sodium succinate. Two modes of tissue work-up were investigated: digestion with subtilisin Carlsberg, a proteolytic enzyme, and homogenization with methanol. The final determination was by reversed-phase high-performance liquid chromatography with dexamethasone as internal standard. The extraction yields of methylprednisolone and dexamethasone from tissue homogenate and the extraction yield of methylprednisolone after incubation with viable tissue were determined. The experiments show that methylprednisolone and the internal standard are extracted in similar yields from tissue homogenates and that methylprednisolone can be recovered in a good yield after incubation with viable tissue, provided that the tissue does not have a high metabolic activity. There was a good agreement between the analytical results from the two different types of tissue work-up. The method of analysis proved feasible for pharmacokinetic work.

INTRODUCTION

Methylprednisolone (MP) is a synthetic derivative of hydrocortisone with enhanced glucocorticoid activity. It can easily be determined in blood or plasma by high-performance liquid chromatography (HPLC) [1–9]. Determination of the steroid in central nervous tissue has been described [4,10]. For the purpose of extensive pharmacokinetic work, we have developed a method for the determination of MP in various types of tissue from rat.

Two modes of work-up of the tissues were compared. The first was digestion with subtilisin Carlsberg, a proteolytic enzyme from *Bacillus subtilis*, which liquifies most tissues [11,12]. The drug is liberated, but during the incubation it is exposed to subtilisin as well as to metabolizing enzymes from the tissue. The second method of work-up consisted of homogenization of the sample in methanol, which rapidly inactivates all enzymes. In both modes of work-up, the free

0378-4347/87/$03.50

fatty acids were removed by means of an alkaline aqueous phase, and the final determination of MP was by reversed-phase HPLC.

EXPERIMENTAL

Reagents and chemicals

Methylprednisolone sodium succinate (MPHS) was a gift from Upjohn (Partille, Sweden). MP was European Pharmacopoeia quality. Dexamethasone (Dx) was purchased from Sigma (St. Louis, MO, U.S.A.). MPHS was dissolved in water and only freshly prepared solutions were used. MP and Dx were dissolved in methanol, and the stock solutions were stable for at least four months in the refrigerator. Subtilisin was purchased from Boehringer-Mannheim Scandinavia (Bromma, Sweden). Acetonitrile (30; E. Merck, Darmstadt, F.R.G.), methanol (45102; BDH, Poole, U.K.) and ethyl acetate (10108; BDH) were used without further purification. The water was distilled and collected in glass.

Animals and samples

Male Wistar rats, weighing 260 ± 40 g, were used for the experiments. The rats were anaesthetized with sodium pentobarbital (60 mg/kg) intraperitoneally. Both femoral arteries and one of the femoral veins were cannulated with polyethylene (PE-90) catheters with a tapered 40-mm tip. The arterial catheters were introduced into the abdominal aorta and the venous catheter into the inferior caval vein. All catheters were exteriorized at the neck. After surgery the rats were kept in specially prepared cages where they had free access to food and water.

The experiment was carried out on awake rats the day after cannulation. The cages were covered in order to avoid disturbing the rats. MPHS (Solu-Medrone®, Upjohn) at a concentration of 50 mg/ml was injected as a single dose of 200 mg/kg bodyweight intravenously over 60 s. The rats were sacrificed after 10, 20, 40, 80, 120 or 160 min by an intravenous injection of a saturated solution of magnesium sulphate.

Immediately after sacrifice tissue samples, typically 200 mg, were taken, in the following order: from liver, right and left kidney, adrenals, small intestine, large intestine, right and left lung, heart, spleen, pancreas, stomach, muscle and brain. The tissue pieces were immediately frozen on dry ice, weighed and stored frozen ($-20°C$) until assayed. The remaining parts of the organs, except for muscle, were dissected, frozen and weighed. The stomach and the intestines were cleared from contents before weighing.

Blank samples were obtained in the same way from untreated rats.

Instrumentation and chromatographic conditions

The liquid chromatography system consisted of an LDC/Milton Roy (Riviera Beach, FL, U.S.A.) Constametric III pump, a Rheodyne 7125 loop injector and an LDC Spectro Monitor III variable-wavelength UV detector. A LiChroCart RP-18 7-μm column (250×4 mm I.D.) was used in conjunction with a Hibar precolumn (30×4 mm I.D.) filled with Perisorb RP-18 (all from Merck). The mobile phase was acetonitrile–0.01 *M* phosphate buffer, pH 7.4 (35:80). The

flow-rate was 2.0 ml/min and the detection wavelength was 240 nm. The injection volume was 10–20 μl.

Tissue work-up by digestion with subtilisin

To weighed, frozen tissue samples, typically 200 mg, were added 1.0 ml of distilled water, 0.2 ml of Tris buffer (1 *M*, pH 10) and an appropriate amount of internal standard (Dx solution). The tissues were homogenized, during cooling with ice, by means of an Ultra-Turrax homogenizer (Janke & Kunkel, Steuren im Breisgau, F.R.G.). A freshly prepared solution of subtilisin in water (10 mg/ml, 0.10 ml) was added and the samples were incubated, with magnetic stirring for 1 h at 55 °C. The liquidized samples were extracted with 3.0 ml of ethyl acetate, and centrifuged at 1000 *g*. The organic phases were transferred to other tubes, and the solvent was evaporated on a water-bath (30 °C) under a stream of dry air. The residues were taken up in 0.1 or 0.3 ml of mobile phase. The mixtures were kept for at least 1 h in the refrigerator for traces of fat to settle at the bottom of the tube before the injection of sample into the chromatograph.

Stability of methylprednisolone and dexamethasone during incubation with subtilisin

The 100-μl samples of MP (100 μg/ml) were diluted with 1.0 ml of distilled water. Then 0.20 ml of Tris buffer (1 *M*, pH 10) and 0.10 ml of subtilisin (6 mg/ml in water) were added. Duplicate samples were incubated, with magnetic stirring, for 0, 1 and 2 h at 55 °C. Then 200 μl of Dx (50 μg/ml) were added, and the samples were extracted with 3.0 ml of ethyl acetate. The residues on evaporation were taken up in 0.3 ml of mobile phase and injected. Dx was treated in the same way, with MP as the internal standard.

Extraction yields of methylprednisolone and dexamethasone from tissue homogenate

Tissue samples, ca. 0.4 g, two from each of the fourteen organs, were homogenized in 1.0 ml of distilled water, with 0.2 ml of added Tris buffer (1 *M*, pH 10). Then 50 μl of MP (200 μg/ml) were added, and the samples were incubated with 2 mg of subtilisin, as above. The samples were extracted with 4.00 ml of ethyl acetate. Of the upper phase, 2.00 ml were added to 100 μl of Dx (50 μg/ml). The solvents were evaporated and the residues were taken up in 0.30 ml of mobile phase. The samples were kept in the refrigerator before injection.

The experiment was repeated with Dx in the incubation mixture and MP as standard.

Extraction yield of methylprednisolone on incubation with viable tissue

Perfadex® tissue perfusion solution (Pharmacia, Uppsala, Sweden) was brought to pH 7.4 with sodium bicarbonate solution and spiked with MP to a concentration of 50 μg/ml. Samples of 0.30 ml of this mixture were warmed to 37 °C. Tissue pieces, ca. 0.4 g, of each of the fourteen organs, were quickly removed from a freshly killed rat, lacerated with a scalpel and put into the samples. After incubation for 10 min at 37 °C, as much as possible of the supernatant from each

sample was sucked off and transferred to a tube containing 200 μl of Dx (50 μg/ml). The same amount of dexamethasone was added to the remaining tissue before homogenization. All samples were then incubated with subtilisin in Tris buffer and analysed as above. The extraction yields were calculated as: yield $= MP_t/(MP_{tot} - MP_{sup})$ where MP_t is the amount of methylprednisolone recovered from the tissue samples, MP_{tot} is the total amount added to the incubation mixture and MP_{sup} is the amount found in the supernatant.

Tissue work-up by extraction with methanol

To weighed, frozen samples, typically 200 mg, were added 2–3 ml of methanol, and an appropriate amount of internal standard (Dx solution). The tissues were homogenized, during cooling with ice, by means of an Ultra-Turrax homogenizer. After centrifugation at 1000 *g*, the clear supernatants were transferred to other tubes, and the volatiles were evaporated on the water-bath under a stream of dry air. The residues were taken up in 3.0 ml of ethyl acetate, and 1.0 ml of sodium hydroxide solution (0.01 *M*) was added. The samples were immediately shaken and centrifuged, the upper phases were transferred to other tubes, and the solvent was evaporated as above. The residues were taken up in 0.5–1.0 ml of mobile phase. The mixtures were kept for 1 h in the refrigerator for traces of fat to settle at the bottom of the tube before the injection of sample into the chromatograph.

Extraction yields of methylprednisolone and dexamethasone from tissue on homogenization in methanol

Tissue samples, ca. 0.4 g, two from each of the fourteen organs, were spiked with 10 μg of MP or Dx and homogenized as above. On centrifugation, the supernatant was transferred to another tube, and the tissue debris was washed with 1 ml of methanol and centrifuged. The second supernatant was added to the first, and the solvent was evaporated. The residues were taken up in 4.00 ml of ethyl acetate, and 1.0 ml of sodium hydroxide solution (0.01 *M*) was added. The samples were immediately shaken and centrifuged. Of the organic phase, 2.00 ml were added to 5 μg of internal standard, Dx or MP, and the solvent was evaporated. The residues were taken up in mobile phase and analysed.

Stability of methylprednisolone sodium succinate during work-up

Tissue samples, ca. 0.2 g, from each organ, were spiked with 40 μg of MPHS and 5 μg of Dx and worked-up by extraction with methanol. The amount of MP formed during work-up and extraction was quantitated in the normal way.

Standard curves and precision

Samples, 0.2 g, of muscle tissue were spiked with 3.13, 6.25, 12.5, or 25 μg of MP (duplicate samples, with blanks), and 25 μl of Dx (200 μg/ml) was added as internal standard. The samples were worked-up by digestion with subtilisin and analysed as described above. A standard curve was drawn, and peak heights were used for quantitation.

Eight 0.2-g samples of muscle tissue were spiked with 2.0 μg of MP, worked-up by digestion with subtilisin and analysed with 5.0 μg of Dx as internal standard.

A rat was given MPHS in the usual way and was sacrificed after 80 min. Seven samples of muscle tissue were analysed on digestion with subtilisin.

Another rat was treated in the same way and eight samples of liver tissue were analysed with homogenization in methanol.

Comparison of the two modes of tissue work-up

Two rats were given the usual dose of MP sodium succinate and were sacrificed after 10 and 40 min. Two tissue samples were taken from each of the fourteen organs, except the adrenals. One set of samples was analysed with subtilisin digestion and the other by extraction with methanol. For each organ, the ratio of the concentration of MP found on subtilisin digestion to the one found on methanol extraction was calculated.

RESULTS

Chromatograms

Representative chromatograms are shown in Figs. 1 and 2. The capacity factor (k') of MP is 8.0 and of Dx 9.4. Peaks that are probably due to metabolites of MP appear at k' 4.8 (in liver incubates) and at k' 4.8, 5.1 and 8.6 (in kidney incubates). When pieces of kidney are analysed by the standard procedures, the first two metabolite peaks are generally higher and the last one is considerably lower.

Stability of methylprednisolone and dexamethasone during incubation with subtilisin

The amount of MP in the incubation mixtures decreased by 5% over 1 h and by 8% over 2 h. There was a similar slight loss of Dx, 5% over 1 h and 6% over 2 h. Under the same conditions, MPHS was completely degraded to MP.

Extraction yields of methylprednisolone and dexamethasone from tissue homogenates

The extraction yields of the steroids on incubation with subtilisin in tissue homogenate are given in Table I.

Extraction yield of methylprednisolone on incubation with viable tissue

The extraction yields of MP on incubation with viable tissue, relative to internal standard added after this incubation, are given in Table I. The very low yields from liver and kidney tissue are probably due to extensive metabolism, which gives rise to large peaks from the metabolites (Fig. 2). The results of liver and kidney incubation were similar when the tissues were worked-up by homogenization in methanol.

Extraction yields of methylprednisolone and dexamethasone from tissue on homogenization in methanol

The extraction yields of the steroids by this mode of work-up are given in Table I.

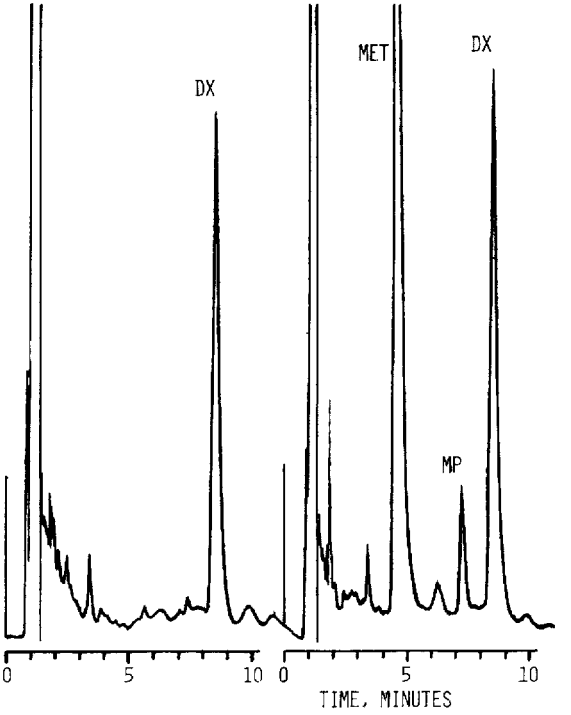

Fig. 1. Chromatograms from tissue samples, after work-up with subtilisin digestion. The first sample is stomach from an untreated rat, while the second is from a rat sacrificed 2 h after an injection of MPHS (200 mg/kg). The tissue concentration is 5.1 μg/g (1.3 μg in the sample). Injected volume, 10 μl; detector, 0.02 a.u.f.s.; recorder, 10 mV, 5 mm/min. Peaks: DX = dexamethasone, internal standard, 5 μg per sample; MET = a metabolite of methylprednisolone (cf. Fig. 2); and MP = methylprednisolone.

Stability of methylprednisolone sodium succinate during work-up

During work-up from tissue homogenates in methanol, there was a maximum degradation of 0.6% of the sodium succinate ester to free MP.

Standard curves and precision

The standard curve was linear with $r = 0.9998$. The eight samples that were spiked with 2.0 μg of MP (ca. 10 μg/g tissue) showed a mean (±S.D.) value of 1.95 ± 0.053 μg, which gives a coefficient of variation (C.V.) of 2.7%.

The seven samples of muscle tissue from the treated rat showed a mean value of 13.0 ± 0.56 μg/g, which gives a C.V. of 4.3%.

The eight samples of liver tissue showed a mean value of 88.8 ± 4.8 μg/g, which gives a C.V. of 5.3%.

Comparison of the two modes of tissue work-up

In the rat that was sacrificed after 40 min, the found concentrations of MP ranged from 105 to 368 μg/g in all organs except the brain, which had a concentration of 8 μg/g by the subtilisin digestion method and of 8.4 μg/g by the methanol extraction method. For all the samples, the mean ratio of found values was

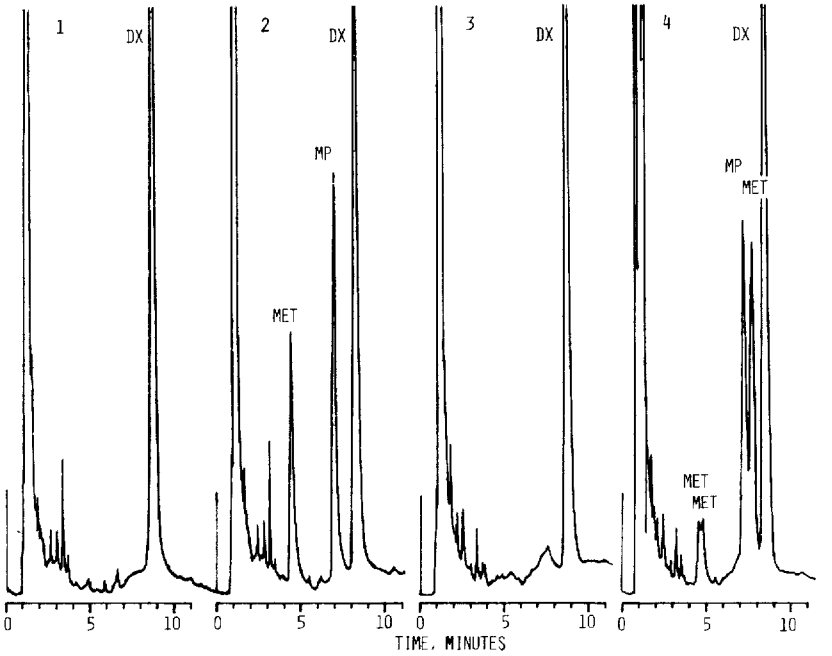

Fig. 2. Chromatograms from incubations of MP for 10 min with organ pieces in Perfadex solution. Work-up by subtilisin digestion. (1) Blank incubation with liver. (2) Incubation of 15 μg of MP with liver. (3) Blank incubation with kidney. (4) Incubation of MP with kidney. Injected volume, 20 μl; detector and recorder settings as in Fig. 1. Peaks: DX = dexamethasone; MET = metabolites of methylprednisolone; MP = methylprednisolone.

0.995, with a standard deviation of 0.125. A plot according to Eksborg [13] gave a uniform scatter around the "1.0"-line. There was consequently no systematic difference between the values from the two methods.

In the rats that were sacrificed after 10 min, the mean concentrations ranged between 4.4 and 425 μg/g. For all samples, the mean ratio of the values was 1.106, with an S.D. of 0.169. This is almost significantly ($0.05 < P < 0.1$, two-tailed t-test) above 1.00, and suggests that the values on subtilisin digestion may have been added to by the presence of MPHS in the tissues.

Tissue concentrations of methylprednisolone

The observed tissue concentrations of MP are given in Fig. 3. The minimum concentration that could be satisfactorily quantitated varied between tissues, due to different patterns of endogenous interferences. In general, the limit of quantification was ca. 1 μg/g.

DISCUSSION

Determination of MP in central nervous tissue has been described [4,10]. In these works, the extraction yields, reported as 70% [4] or more than 98% [10],

TABLE I

EXTRACTION OF METHYLPREDNISOLONE (MP) AND DEXAMETHASONE FROM TISSUE HOMOGENATE AND EXTRACTION OF MP ON INCUBATION WITH VIABLE TISSUE

Organ	Absolute extraction yields from homogenate (%)				Extraction yield of MP, relative to internal standard, from viable tissue (%)
	Methylprednisolone		Dexamethasone		
	Subtilisin	Methanol	Subtilisin	Methanol	
Liver	44*	83	73	94	38*
Right kidney	40*	88	76	93	7*
Left kidney	44*	85	77	92	8*
Adrenals	74	88	73	90	82
Small intestine	76	88	77	98	85
Large intestine	87	88	74	98	73
Right lung	73	65	59	90	89
Left lung	74	75	58	81	89
Heart	77	58	75	88	90
Spleen	66	85	69	93	94
Pancreas	54	82	57	98	88
Stomach	64	82	56	100	79
Muscle	70	83	88	97	92
Brain	82	78	79	94	88

*There were peaks due to metabolites in the chromatograms.

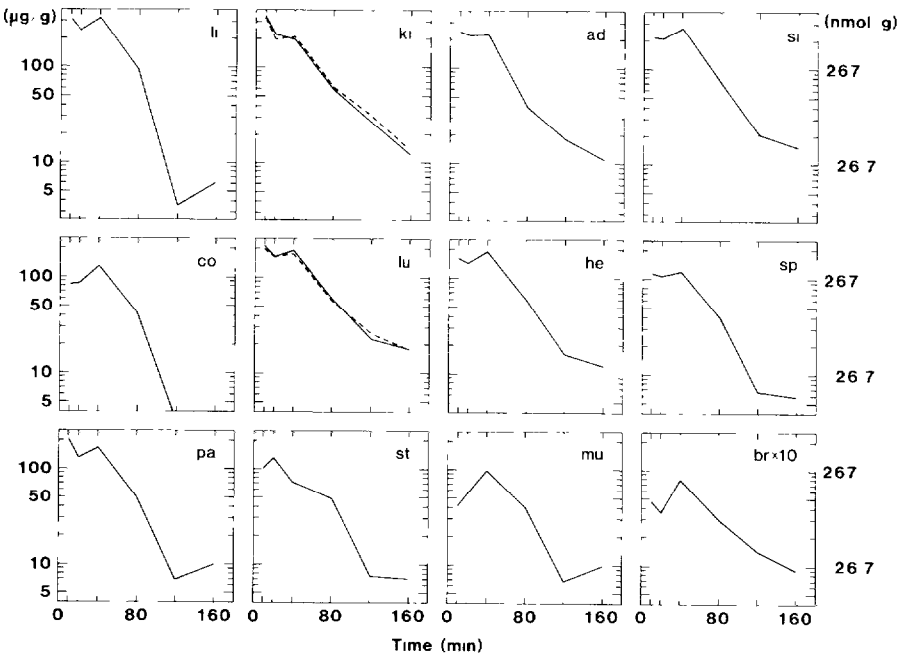

Fig. 3. Median concentrations ($n = 5$–9) of methylprednisolone in various rat tissues (li = liver; ki = left and right kidney; ad = adrenals; si = small intestine; co = colon; lu = left and right lung; he = heart; sp = spleen; pa = pancreas; st = stomach; mu = muscle; br = brain). The concentrations of MP in the brain are multiplied by ten.

were determined on addition of steroid to homogenized tissue. This procedure does not, however, mimic the uptake of drug into tissue in the living animal. After drug uptake under physiological conditions, the extraction yields from the tissue may be lower. The drug may have penetrated into the cells to become bound to structures from which it is not easily liberated. With simple solvent extraction of the tissue homogenate, part of the analysed drug may remain trapped in particulate matter of the denatured tissue, while the internal standard to a greater extent remains free in the solution.

Radioactively labelled drug can be used to determine extraction yields only if the drug is not metabolized. Otherwise the amount of radioactivity remaining in the tissue will represent the sum of unextracted, unchanged drug and unextracted metabolites. Since MP is readily metabolized [14], we chose not to use labelled drug but to gather indirect evidence that our analytical results reflect the actual amount of MP in the tissue samples. The experiments show that MP and the internal standard are extracted in similar yields from tissue homogenate and that MP can be recovered in a good yield after incubation with viable tissue, provided that the tissue does not have high metabolic activity. As expected from the properties of the enzyme [11,12], MP and Dx, but not the hemisuccinate ester, were stable towards subtilisin under the conditions used. The good agreement between the analytical results after two wholly different types of work-up gives an additional indication that the extraction yields are satisfactory in both cases. The extensive metabolism observed in the liver and kidney incubations shows that the tissues were indeed viable and that MP penetrated into them during the incubation.

In spite of what should be an unfavourable pH and temperature, some metabolism of MP also took place during the digestion of liver and kidney samples with subtilisin (Table I). The extent of this metabolism varied considerably, and, with rapid heating of the incubation mixtures to their final temperature, it could sometimes be avoided. However, of the two modes of extraction, the homogenization in methanol is recommendable for liver and kidney samples.

The precision of the method had to be tested in two different ways. The use of spiked samples permitted an estimation of the accuracy and precision of the HPLC method itself, since the correct amount of MP in the sample was known. The use of several samples from treated rats permitted an estimation of precision that included the variance due to the weighing of the tissue pieces. Since the "true" concentration of steroid in these samples was not known, accuracy could not be estimated. The results of the tests were considered as satisfactory.

MP is not used as such in parenteral formulations, but as the very soluble sodium succinate, a hemiester. This pro-drug is rapidly hydrolysed in vivo, with a half-life of ca. 10 min in rabbits [7] as well as in our rats [15]. It is also poorly distributed into tissue [15]. During the first 30 min after injection, however, significant amounts of it may be present in the tissue samples. It should therefore be noted that, since the alkaline subtilisin solution hydrolyses the ester, determination of MP by means of subtilisin digestion measures the sum of MP and MPHS. In contrast, during homogenization with methanol, all endogenous enzymes are inactivated and none is added, and the hemisuccinate present remains intact and will then be removed by the alkaline aqueous phase. Consequently, the

analytical method in itself distinguishes between MP and MPHS. However, rapid hydrolysis can be expected to occur during the dissection of the animal, and it is therefore questionable whether any analysis can make a true distinction between the two steroids in tissue samples.

Since all tissues, apart from liver and kidney, showed approximately similar behaviour in the analysis, we chose to consider "tissue" as a single type of biological matrix. A special study on extraction recovery, detection limit and sensitivity on each of the fourteen organs would require a rather formidable amount of experimental work. When judging the acceptability of this generalization, it must also be borne in mind that not one of the organs in itself represent a homogenous biological matrix with a single, homogenous concentration of MP. With these objections well in mind, we consider that we have developed a feasible method for the quantitation of MP in rat tissue at the resolution level of the entire organ.

In conclusion, we have explored two ways of extraction and quantitation of MP in rat tissue. The good correlation between results obtained with the two procedures indicates that the yields of extraction are satisfactory.

ACKNOWLEDGEMENTS

This work was supported by Tore Nilsons Fund for Medical Research. Our thanks are due to Mrs. Gertie Jönsson for skilful technical assistance.

REFERENCES

1 D.C. Garg, J.W. Ayres and J.G. Wagner, Res. Commun. Chem. Pathol. Pharmacol., 18 (1977) 137.
2 J.Q. Rose and W.J. Jusko, J. Chromatogr., 162 (1979) 273.
3 M. Dix Smith, J. Chromatogr., 164 (1979) 129.
4 P.A. McGinley, J.M. Braughler and E.D. Hall, J. Chromatogr., 230 (1982) 29.
5 W.F. Ebling, S.J. Szefler and W.J. Jusko, J. Chromatogr., 305 (1984) 271.
6 H. Derendorf, H. Möllmann, P. Rohdewald, J. Rehder and E.W. Schmidt, Clin. Pharmacol. Ther., 37 (1985) 502.
7 W.F. Ebling, S.J. Szefler and W.J. Jusko, Drug. Metab. Dispos., 13 (1985) 296.
8 G.J. Lawson, J. Chromatogr., 342 (1985) 251.
9 J.A. Shah and D.J. Weber, J. Chromatogr., 344 (1985) 41.
10 J.M. Braughler and E.D. Hall, J. Neurosurg., 58 (1983) 538.
11 M.D. Osselton, Methodol. Surveys Biochem., 10 (1981) 101.
12 M.D. Hammond and A.C. Moffat, J. Forensic Sci. Soc., 22 (1982) 293.
13 S. Eksborg, Clin. Chem., 27 (1981) 1311.
14 T. Mineo, Folia Endocrinol. Jpn., 52 (1976) 1243.
15 M. Jonung, J. Gabrielsson, J. Idvall and S. Björkman, unpublished results.