A METHOD FOR THE ESTIMATION OF SOME SYNTHETIC GLUCOCORTICOSTEROIDS IN RAT MUSCLE*

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SUMMARY

A gas–liquid chromatographic method has been developed for the estimation of the concentrations of some synthetic glucocorticosteroids in muscle after administration of these compounds to rats. The method depends on the preparation of the trimethylsilyl ethers of the corticosteroids by the use of a mixture of the silylating reagents N,O-bis(trimethylsilyl)acetamide, trimethylsilylimidazole and trimethylchlorosilane. Mass spectrometry has indicated that the use of this technique results in the formation of the tautomeric enol trimethylsilyl ethers of the hydroxyl groups. The derivatives are stable on gas–liquid chromatography and are measured by electron-capture detection. Using this method it was possible to estimate triamcinolone acetonide, triamcinolone, betamethasone and prednisolone in muscle after administration of doses of 20 mg/kg body weight.

INTRODUCTION

Previous work carried out in these laboratories into the catabolic effects on skeletal muscle of the administration of glucocorticosteroids[1,2] indicated the need for a method for the estimation of the concentrations of these steroids in muscle. The compounds of greatest interest were triamcinolone acetonide, triamcinolone, dexamethasone, betamethasone and prednisolone. Various methods[3-10] which have been applied to the measurement of corticosteroids in biological tissue were considered but none appeared to provide the required specificity and sensitivity.

It was found that the use of a mixture of the silylating reagents N,O-bis(trimethylsilyl)acetamide, trimethylsilylimidazole and trimethylchlorosilane resulted in the preparation of derivatives of triamcinolone, betamethasone and prednisolone which were stable under the chromatographic conditions employed. The response of the electron-capture detector to these derivatives was approximately three times

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* Proper names of steroids: betamethasone = 9α-fluoro-16β-methyl-11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione; cortisol = 11β,17α,21-trihydroxypregn-4-ene-3,20-dione; corticosterone = 11β,21-dihydroxypregn-4-ene-3,20-dione; dexamethasone = 9α-fluoro-16α-methyl-11β,-17α,21-trihydroxypregna-1,4-diene-3,20-dione; prednisolone = 11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; triamcinolone = 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione; triamcinolone acetonide = 9α-fluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxy-pregna-1,4-diene-3,20-dione; 6β-hydroxy-triamcinolone acetonide = 9α-fluoro-6β,11β,21-tri-hydroxy-16α,17α-isopropylidenedioxypregna-1,4-diene-3,20-dione.

greater than that of the flame ionisation detector. It was found to be sufficient to allow the estimation of submicrogram quantities of these steroids in muscle after extraction and a more simple purification of the extract than would be required if the flame ionisation detector were to be used.

EXPERIMENTAL

Materials

Triamcinolone acetonide and triamcinolone were obtained from E.R. Squibb and Sons Ltd., Liverpool, Great Britain, prednisolone from CIBA Laboratories, Horsham, Great Britain, dexamethasone from Merck, Sharp and Dohme, Ltd., Hoddesdon, Herts., Great Britain, betamethasone from Glaxo Laboratories Ltd., Greenford, Middlesex, Great Britain, and corticosterone and cortisol from Steraloids Ltd., Croydon, Great Britain. The following labelled compounds were all obtained from C.E.N. Radioisotopes Department, Mol-Donk, Belgium: [1,2,4-^{3}H$_3$]triamcinolone acetonide, specific activity 9.5 Ci/mmole; [1,2,4-^{3}H$_3$]triamcinolone, 5.0 Ci/mmole; [1,2,4-^{3}H$_3$]dexamethasone, 5.6 Ci/mmole; [1,2,4-^{3}H$_3$]betamethasone, 5.0 Ci/mmole; [1,2-^{3}H$_2$]prednisolone, 12.5 Ci/mmole. [1,2-^{3}H$_2$]corticosterone (specific activity 36 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, Great Britain.

Unlabelled steroids were dissolved in tetrahydrofuran to give solutions with concentrations of 1 mg/ml and were stored at 4°. The purity of the solutions was checked by thin-layer chromatography (TLC). Standard solutions were diluted as required in tetrahydrofuran; diluted solutions were kept at 4° for not longer than one month. Tritiated steroids were diluted with tetrahydrofuran to a concentration of 100 μCi/ml. These solutions were stored at 4° and were checked for purity by TLC. The solutions were diluted to a concentration of 0.5 μCi/ml for use as internal standards in the measurement of the recovery of steroids from tissue samples.

N,O-Bis(trimethylsilyl)acetamide (BSA), N-trimethylsilylimidazole (TMSI) and trimethylchlorosilane (TMCS) were obtained from Pierce Chemical Co., Rockford, Ill., U.S.A. 2,5-Bis-(5-tert.-butyl-benzoxazol-2-yl)-thiophene (BBOT) was obtained from CIBA (A.R.L.), Cambridge, Great Britain. Scintillation fluid was prepared by adding Methyl Cellosolve (1.7 l), BBOT (30 g) and naphthalene (340 g) to toluene (2.5 l). Tetrahydrofuran was purified by heating under reflux with K, for 2 h, followed by double distillation. Ethyl acetate was purified by washing with one volume of 5% (w/v) aqueous NaHCO$_3$, followed by distillation over solid CaO. The solvent was stored over anhydrous Na$_2$SO$_4$. Methanol was purified by heating under reflux with 2,4-dinitrophenylhydrazine and concentrated HCl for 3 h, followed by double distillation. Hexane was purified by redistillation from anhydrous Na$_2$SO$_4$ and was stored over anhydrous Na$_2$SO$_4$.

"Supasorb" (100–120 mesh) was obtained from BDH Ltd., Poole, Great Britain. It was acid-washed and silanised with dichlorodimethylsilane. Excess and partially reacted silanising agent was removed by washing with n-propanol. OV-17 was obtained from Applied Science Laboratories, Inc., State College, Pa., U.S.A. The column packing of 1% (w/w) OV-17 on "Supasorb" was prepared by the drying-on technique, using a solution of OV-17 in hexane. Columns were packed under nitrogen at a pressure of 20 lb./sq. in.

Nitrogen (high purity) was obtained from Air Products Ltd., Hythe, Southampton, Great Britain.

Equipment

A Pye Model 84 gas chromatograph containing a 10-mCi ^{63}Ni source in the electron-capture detector was obtained from Pye Unicam Ltd., Cambridge, Great Britain. The mass spectrometer used was MS9 (A.E.I.-G.E.C.). An Ultra-Turrax homogeniser was supplied by Janke and Kunkel KG, Staufen i. Dr., G.F.R. and "Reacti-Vials" were obtained from Pierce Chemical Co.

Glassware was cleaned by soaking it in detergent, rinsing, soaking in chromic acid overnight followed by repeated rinsing in tap water and then distilled water. It was oven-dried and silanised with dichlorodimethylsilane.

Animals

Albino Wistar rats (250–300 g) CFHB strain were obtained from Carworth Europe, Alconbury, Huntingdon, Great Britain.

Methods

Preparation of trimethylsilyl ethers of steroids. Appropriate volumes of the steroid solutions were pipetted into the reaction vials. The solvent was removed by evaporation in a stream of nitrogen. BSA (20 μl), TMSI (20 μl) and TMCS (10 μl) were added to the closed reaction vials by syringe. The vials were shaken and left at room temperature overnight.

Gas chromatographic operating conditions. Glass columns, 9 ft. $\times$ 1/4 in. O.D. were used, with OV-17 (1 %, w/w) on "Supasorb" as the stationary phase. The column oven temperature was 260° and the detector temperature 350°. The pulse interval was 150 μsec. As carrier gas nitrogen was used at a flow-rate of 100 ml/min.

Samples were applied by the "on-column" injection technique. The injection port was maintained at 265°. The column oven temperature was reduced to 200° overnight to minimise column bleeding effects.

Peak areas were calculated as the product of the peak height and the width of the peak at half the peak height.

Administration of steroids and removal of tissue. Steroids were injected intraperitoneally as fine suspensions in 0.5 ml of 0.9 % (w/v) aqueous NaCl. Control animals received saline only. Animals were killed by decapitation and tissues removed immediately. The skeletal muscles used were the *vastus lateralis, vastus medialis* and *gluteus medius.* The heart was removed intact and washed quickly to remove clotted blood. All tissues were frozen immediately in solid CO_2 and stored at $-20°$.

Extraction of steroids from tissues. Tissue samples (approximately 4 g of skeletal muscle and 1 g of heart) were finely chopped, the skeletal muscles being pooled, and placed in large centrifuge tubes to which aliquots of the tritiated form of the steroid being measured had been added as internal standards for the calculation of losses during the extraction procedure. The activity of the labelled steroids was such that the internal standard contributed less than 1 ng to each muscle sample. Equivalent aliquots of the tritiated steroid had been placed in vials for scintillation counting. A saturated aqueous solution of sodium sulphate (32 ml) was added to each tube and the tissue homogenised with an Ultra Turrax homogeniser for 2 min

at 8000 r.p.m. During homogenisation the tube containing the tissue was held in iced water. The suspension was then extracted twice with 125 ml of ethyl acetate. The extracts were combined and the solvent removed by rotary evaporation at 25°. The residue was then transferred, with 16 ml of ethyl acetate, to a separating funnel. where it was washed successively with 2 ml each of aqueous NaOH (0.1 N), acetic acid (0.5 %, v/v) and distilled water. All these solutions were saturated with solid Na_2SO_4. The organic phase was transferred to an evaporating flask and the ethyl acetate removed by rotary evaporation at 25°. The extract was further purified by partitioning, twice, between hexane (1 ml) and 70 % aqueous methanol (4 ml). The hexane layer was discarded and the aqueous methanol removed by evaporation under a stream of nitrogen at 25°. The residue was dissolved in tetrahydrofuran and transferred to the reaction vial. A suitable quantity, usually 0.5–2 μg, of the internal standard for gas–liquid chromatography (GLC) was placed in each reaction vial. The tetrahydrofuran was removed in a stream of nitrogen, at room temperature. After silylation, aliquots (5 μl) were withdrawn from the vials, using a micro-syringe, for scintillation counting, in order to determine recovery of the tritiated internal standard.

RESULTS

Chromatography of corticosteroid derivatives

The retention times together with the response factors of the corticosteroid trimethylsilyl ethers are shown in Table I.

The process of silylation apparently resulted in the formation of a single derivative of each steroid except in the case of dexamethasone. Only one symmetrical elution peak was seen by chromatography, using the electron-capture detector, of trimethylsilyl ethers of triamcinolone acetonide, triamcinolone, prednisolone, betamethasone, corticosterone and cortisol (Figs. 1a and 1b). Chromatography of dexamethasone trimethylsilyl ether resulted in the separation of two compounds with retention times of 18.4 min (minor peak) and 22.2 min (major peak). The ratio

TABLE I

RETENTION TIMES OF CORTICOSTEROID TRIMETHYLSILYL ETHERS AND RESPONSE FACTORS FOR THE ELECTRON-CAPTURE DETECTOR

Conditions for GLC as described in *Methods*. Response factors and relative retention times quoted are relative to that of the derivative of triamcinolone acetonide. The area of the peak produced by injection of 20 ng of triamcinolone acetonide as the trimethylsilyl ether was 870 mm² at 5×10^{-10} A f.s.d. The figures for the response factors represent the mean of five experiments, with the standard deviation.

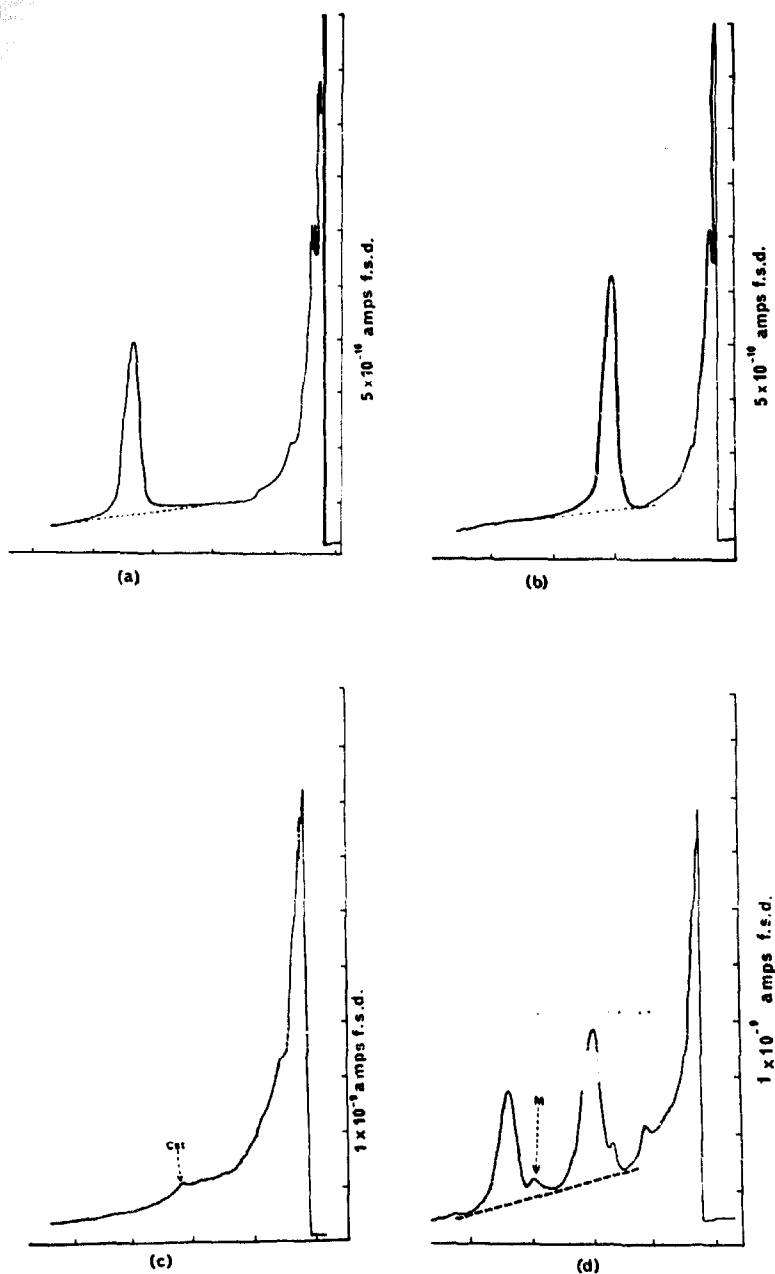| Steroid	Retention time (min)	Relative retention time	Relative response factor
Triamcinolone acetonide	31.5	1.00	1.00
Triamcinolone	23.6	0.75	2.21 ± 0.15
Prednisolone	17.9	0.57	0.69 ± 0.06
Corticosterone	19.5	0.62	0.14 ± 0.05
Cortisol	16.7	0.53	0.47 ± 0.09
Betamethasone	20.8	0.66	2.13 ± 0.11

Fig. 1. Tracings of chromatograms of (a) triamcinolone acetonide trimethylsilyl ether (10 ng); (b) prednisolone trimethylsilyl ether (20 ng); (c) silylated extract of muscle (4 g) from untreated rat; (d) silylated extract of rat muscle (4 g) removed 2 h after administration of a 20 mg/kg dose of triamcinolone acetonide. Prednisolone (1 µg) was added to the extract as internal standard. In each case the sample was treated and chromatographed as described in *Methods*. Cst = Endogenous corticosterone; M = metabolite of triamcinolone acetonide. Chart speed, 6 in./h.

of the major to the minor peak was increased by carrying out the silylation reaction at 60° but it was not possible to eliminate the smaller peak completely and therefore it was not possible to apply the method to the estimation of dexamethasone.

Both TLC and the removal of the silylating reagents from the derivatives led to their breakdown so that it was necessary to use an indirect method to assess the degree of conversion of the corticosteroids. The silylation was carried out on prednisolone and triamcinolone acetonide together in varying quantities. These are listed in Table II, together with the results. The ratio of the corrected peak areas of the two compounds is proportional to the ratio of the concentration of each steroid in the reaction vial. This relationship applies over a range for triamcinolone acetonide of 2 ng to 60 ng injected, and for prednisolone of 4 ng to 80 ng injected. Injection of larger quantities of either steroid did not produce a relative increase in peak area, due to saturation of the detector current.

TABLE II

RESPONSE OF THE ELECTRON-CAPTURE DETECTOR TO DIFFERENT AMOUNTS OF PREDNISOLONE TRIMETHYLSILYL ETHER AND TRIAMCINOLONE ACETONIDE TRIMETHYLSILYL ETHER

Pr = Prednisolone trimethylsilyl ether; TA = triamcinolone acetonide trimethylsilyl ether. Conditions as described in *Methods*. The relative response factor Pr/TA is 0.69.

Quantity injected (ng)		Ratio TA/Pr	Peak area at $5 \times 10^{-10}A$ f.s.d. (mm²)		Ratio of peak areas TA/Pr	Ratio of peak areas corrected for relative response Pr/TA
Pr	TA		Pr	TA		
20	2	0.10	541	76	0.14	0.10
20	4	0.20	503	154	0.31	0.21
20	10	0.50	970	455	0.68	0.47
20	20	1.00	601	870	1.45	1.00
20	40	2.00	552	1829	3.14	2.16
20	60	3.00	592	2646	4.47	3.05
20	100	5.00	534	3225	6.05	4.17
4	20	5.00	124	882	7.12	4.91
10	20	2.00	288	840	2.92	2.01
20	20	1.00	600	882	1.47	1.01
40	20	0.50	1167	864	0.74	0.51
80	20	0.25	2357	872	0.37	0.26
100	20	0.20	2744	892	0.34	0.23

The coefficient of variation of the ratio of the peak areas, using a sample containing 1 µg of prednisolone and 0.5 µg of triamcinolone acetonide, was ±3% (seven determinations). The coefficient of variation of sampling, injection and detector response was between 5% and 6%. The precision of measurement of the peak areas ratio is thus greater than that of measurement of absolute peak areas.

The relative retention times of the four steroids indicated that prednisolone might be used as an internal standard for the estimation by GLC of triamcinolone acetonide and the latter as the internal standard for the estimation of prednisolone, triamcinolone and betamethasone. Justification for the use of prednisolone and triamcinolone acetonide as internal standards rests in the constancy of the ratio of the response of the electron-capture detector to the steroid being measured and that used as the internal standard. This has been demonstrated for triamcinolone acetonide

and prednisolone (Table II) and applied also to the two pairs triamcinolone and triamcinolone acetonide and betamethasone and triamcinolone acetonide (see Table I).

Mass spectrometry of corticosteroid derivatives

The derivatives formed by silylation of the corticosteroids were analysed by mass spectrometry. It was not possible to carry out the analysis on solid samples owing to the instability of the derivatives. Capillary sample probes were filled with a solution of each derivative in the silylating reagent. The concentration of the steroid in each solution was 20 μg/ml. The probes were placed in the mass spectrometer and the solvent evaporated under vacuum at low temperature. After removal of the solvent the temperature of the sample probe was raised to allow sublimation of the steroid derivative.

In the spectrum of each steroid derivative the ion of highest m/e value which showed the silicon isotope pattern was deemed to be the parent or molecular ion. Table III shows the m/e value of the parent ion and of the base peak for each steroid.

TABLE III

ANALYSIS OF MASS SPECTRA OF CORTICOSTEROID TRIMETHYLSILYL ETHERS

Steroid	Molecular ion (m/e)	Base peak (m/e)	Interpretation
Triamcinolone acetonide	651	447	M-(131 + 73)
Triamcinolone	828	477	M-(side-chain + $C_{15,16,17}$)
Prednisolone	722	445	M-(204 + 73)
Cortisol	724	447	M-(204 + 73)
Corticosterone	635	562	M-73
Dexamethasone	754	537	M-(204 + 15)
Betamethasone	754	461	M-(204 + 90) + 1

The parent ion for each steroid derivative, with the exception of that of triamcinolone acetonide, was of a value which indicated that silylation of all the oxygen functions had occurred; this included the ketone groups at C_3 and C_{20} as well as the hydroxyl groups, where present, at C_{11}, C_{16}, C_{17}, C_{21}. That triamcinolone acetonide is not fully silylated is suggested by the fact that there is no detectable ion at m/e 724, nor at m/e 709, which would indicate the loss of a methyl group from a parent ion of m/e 724, whereas there is an ion, of relative abundance 11% with an m/e value of 651. This is the molecular weight of the tris(trimethylsilyl) ether of triamcinolone acetonide. Evidence for the absence of a trimethylsilyl group on the enol form of the C_{20} ketone of triamcinolone acetonide is provided by the presence of an ion at m/e 520 which indicates loss of a fragment of molecular weight 131. This ion (M-131) is not seen in the spectra of the other steroid derivatives. It is suggested that it is due to loss of the C_{21} trimethylsilyl ether-C_{20} ketone side-chain. The presence of the ion (M-204) in the spectrum of triamcinolone acetonide is attributed to the loss of the side-chain and of a trimethylsilyl group. This fragment (M-204) also appears in the spectra of most of the other compounds and is attributed in these cases to loss of the disilylated side-chain. The presence of an ion at m/e 754 in the spectrum of

dexamethasone indicates that the steroid is converted to the penta(trimethylsilyl) ether. The separation of two compounds by GLC may be due to the formation of a mixture of dexamethasone penta(trimethylsilyl) ether and dexamethasone tetra-(trimethylsilyl) ether. A number of the ions seen in the spectrum could be due to fragmentation of the latter derivative. Line diagrams of the mass spectra of the corticosteroid trimethylsilyl ethers are shown in Fig. 2.
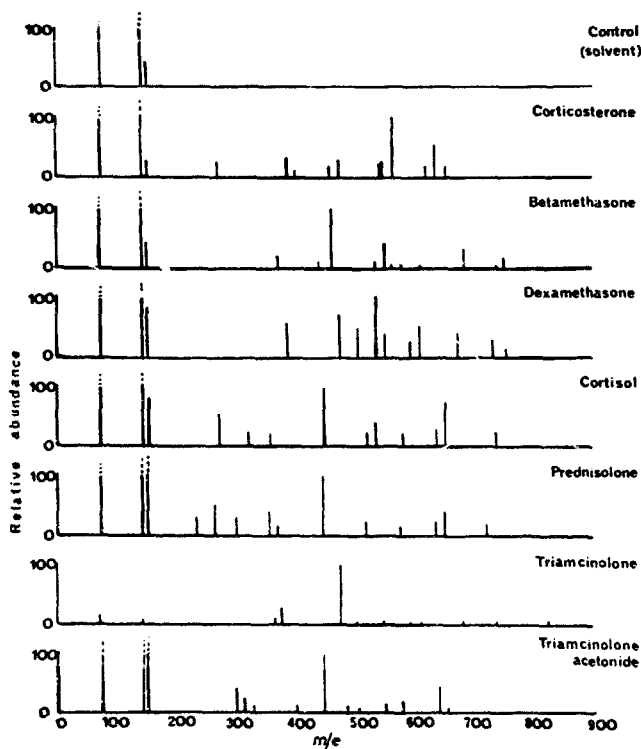

Fig. 2. Line diagram of the mass spectra of corticosteroid trimethylsilyl ethers.

GLC of corticosteroids added to extracts of muscle

In order to establish that quantitative extraction of the steroids from tissue samples could be accomplished and that other components extracted from the samples did not interfere with the measurement of the steroids during gas chromatography the following experiments were performed.

Extracts were made of muscle and heart from control rats. The extracts were treated with silylating reagents and 1-μl aliquots were chromatographed. The chromatograms showed no peaks which would interfere with the measurement of the corticosteroid trimethylsilyl ethers (Fig. 1c). Triamcinolone acetonide, triamcinolone, betamethasone and prednisolone were added to separate extracts of control muscle and the mixtures silylated. Chromatography of these extracts containing the corticosteroid trimethylsilyl ethers showed symmetrical peaks with the same retention times as those of the pure steroid trimethylsilyl ethers.

The same steroids were then added to samples of muscle and heart from control animals, before homogenisation and extraction of the samples. After silylation the mixtures were subjected to GLC. The resulting chromatograms showed that the steroids were recovered unchanged by the extraction method used.

Rats were injected with triamcinolone acetonide, triamcinolone, betamethasone or prednisolone (20 mg/kg) and were killed at 1 or 2 h after injection. A chromatogram of the silylated extract of muscle from a treated animal is reproduced in Fig. 1d. The chromatograms of the extracts of muscle from rats treated with prednisolone, betamethasone and triamcinolone showed no additional peaks but those from muscle from rats treated with triamcinolone acetonide showed a small peak, with a retention time of 27.5 min, which was thought to represent a metabolite of triamcinolone acetonide, 6β-hydroxy-triamcinolone acetonide[11].

Efficiency of recovery of corticosteroids from control muscle

The recovery of the corticosteroids from samples of control muscle by the method of extraction described was examined. For this purpose known amounts of labelled and unlabelled steroids were placed in the tubes used for homogenisation of the muscle. The solvent was removed by evaporation, under nitrogen, at room temperature, before the muscle was added to the tubes. Samples weighing approximately 4 g consisted of muscle from rats which had not been treated with steroids. Two samples of muscle to which no steroid had been added were included to serve as control samples for liquid scintillation counting and for GLC. The samples were extracted, silylated and chromatographed as previously described. The quantities of steroids used and the results obtained are listed in Table IV. The total losses during the procedure, as determined by the loss of tritiated steroids, were between 12 and 39 %. There was no consistent difference in the recovery of the four steroids, nor in recovery from samples containing different amounts of steroids. This indicates that losses occurred as a result of the manipulation of the samples and solvents rather than as a result of processes such as adsorption on glassware or inefficient extraction by the solvents. Had the losses been due to these processes, consistently greater loss would have been expected from samples containing least steroid. Adjustment of the values determined by GLC according to the recovery of the tritiated steroids resulted in corrected recovery figures for the four steroids of between 94 and 108 %. These results indicate that the tritiated steroid is not distinguished from the unlabelled steroid by the processes used and confirm the reproducibility of the silylation method.

The precision of measurement of triamcinolone acetonide concentration in muscle from treated rats was determined. Samples of muscle were taken from five rats to which the steroid had been administered at a dose level of 20 mg/kg. The muscle samples were pooled and finely minced before being divided into five samples. The steroid was extracted from the samples, silylated and chromatographed. The coefficient of variation of the technique was found to be 10 %.

The sensitivity of the method was such as to allow the estimation of tissue levels above 0.01 $\mu g/g$ of triamcinolone acetonide, triamcinolone and betamethasone, and of levels above 0.02 $\mu g/g$ of prednisolone. It was not possible to measure accurately lower concentrations in tissues although lesser quantities of the pure steroids could be measured. The increase in the size of the solvent peak caused by the tissue

TABLE IV

RECOVERY OF CORTICOSTEROIDS FROM SKELETAL MUSCLE

The steroids were added to the samples (approximately 4 g) before homogenisation and extraction.

Amount of steroid added to sample (µg)	Amount of steroid added to muscle determined by GLC (µg)	Recovery of tritiated internal standard (%)	Calculated amount of steroid in original sample (µg)
Triamcinolone acetonide			
0.5	0.34	71	0.48
0.5	0.36	61	0.49
1.0	0.80	82	0.98
1.0	0.72	67	1.08
2.0	1.51	71	2.12
2.0	1.49	74	2.02
Prednisolone			
0.5	0.32	68	0.47
0.5	0.39	79	0.49
1.0	0.83	81	1.02
1.0	0.05	73	0.89
2.0	1.46	74	1.98
2.0	1.11	62	1.80
Triamcinolone			
0.5	0.34	69	0.49
0.5	0.35	73	0.46
1.0	0.83	81	1.02
1.0	0.73	75	0.97
2.0	1.71	88	1.94
2.0	1.75	85	2.00
Betamethasone			
0.5	0.33	70	0.47
0.5	0.31	64	0.48
1.0	0.66	66	1.00
1.0	0.73	71	1.03
2.0	1.59	79	2.02
2.0	1.61	82	1.96

components resulted in a higher baseline than was seen in chromatograms of the pure steroid derivatives, and in increased noise levels on the chromatograms. These two effects reduced the sensitivity of the method to the levels quoted.

Measurement of corticosteroids in skeletal muscle and heart after intraperitoneal administration to rats

The method of estimation by GLC was applied to the determination of the concentrations of triamcinolone acetonide and triamcinolone in skeletal muscle and heart, and of betamethasone and prednisolone in skeletal muscle, after administration of the steroids at 20 mg/kg dose levels. Samples were taken from rats killed at intervals during the period 5 min to 12 h after administration of the drugs. Steroid concentration in a sample of skeletal muscle from each of five rats was estimated separately. The hearts from the five animals were pooled, minced and divided into two portions, for duplicate assay. The figures quoted (Table V) for the concentrations of steroid

in muscle represent the mean of the values found, and the standard deviation is therefore a measure of the variability of tissue concentrations between animals.

This variability is considerably higher than the variability due to the method, which, as already quoted, is 10 %. The variation between the concentrations found in different animals is presumably due to small differences in the size of the animals, to different rates of metabolism of the steroids and to the mode of administration, as a small variation in the site of injection may affect the rate of uptake of the steroid into the blood and thus into the tissues.

Comparison of the levels of triamcinolone acetonide and triamcinolone in skeletal muscle and in heart shows that the concentration in the heart is between two and three times that in skeletal muscle.

DISCUSSION

The development of a method for the determination of triamcinolone acetonide, triamcinolone, betamethasone and prednisolone in rat muscle has been achieved through the formation of the trimethylsilyl ethers of the corticosteroids, these derivatives being suitable for GLC. It has been suggested that in order to produce stable silylated derivatives of corticosteroids it is necessary first to protect the ketone groups at C_3 and C_{20}[10,12,13]. The step has now been shown not to be necessary. Analysis of the derivatives by mass spectrometry has shown that all the hydroxyl and ketone groups of the corticosteroids are converted to the trimethylsilyl ethers, with the exception of the C_{20} ketone of triamcinolone acetonide. This silylation is achieved through the use of a mixture of the reagents BSA, TMSI and TMCS. Klu et al.[14] have demonstrated the silylation by BSA of a cyclic ketone through the formation of the enol tautomer. The mass spectra indicate that the C_{19} methyl is not lost during the silylation reaction and therefore the most likely overall reaction for the formation of the trimethylsilyl ether of the C_3 ketone of the diene compounds is as follows:
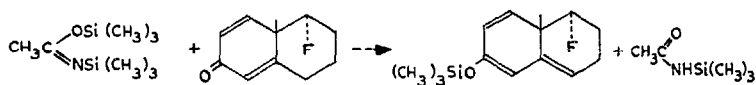
The formation of the trimethylsilyl ether of the C_3 ketone of the mono-ene compounds may be similar or it may be as follows:
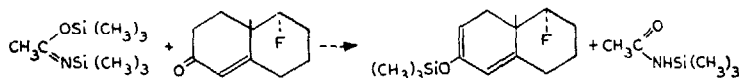
The formation of the trimethylsilyl ether of the C_{20} ketone is as follows:
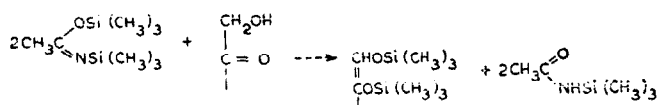
The observation that betamethasone penta(trimethylsilyl) ether is more stable than the dexamethasone derivative on GLC is due to the fact that when the 16-methyl group is *cis* relative to the 17-hydroxyl group it will offer considerable steric

TABLE V

CONCENTRATION OF STEROIDS (μg/g) DETERMINED BY GLC IN SKELETAL MUSCLE AND HEART AFTER ADMINISTRATION AT A DOSE LEVEL OF 20 mg/kg

Values for muscle represent the mean $\pm$ the standard deviation of the concentrations found in five animals. Values for heart represent the mean of the concentration found in duplicate samples consisting of the pooled hearts from five animals. Half-life values quoted were estimated from a log-linear graph of the results. TA = Triamcinolone acetonide; T = triamcinolone; B = betamethasone; Pr = prednisolone.

Steroid	Tissue	Time (min)								Half-life (min)
		15	30	60	90	120	240	360	720	
TA	Skeletal muscle	0.53 ± 0.01	0.48 ± 0.08	0.43 ± 0.05	0.30 ± 0.07	0.20 ± 0.03	0.14 ± 0.03	0.12 ± 0.01	0.05 ± 0.02	158
	Heart	1.44	n.e.[a]	1.86	0.49	0.52	0.34	0.39	n.e.	170
T	Skeletal muscle	0.71 ± 0.15	0.76 ± 0.11	0.96 ± 0.42	1.24 ± 0.32	0.67 ± 0.05	0.15 ± 0.03	<0.01	n.e.	59
	Heart	1.33	1.70	1.86	1.64	1.13	0.43	<0.01	n.e.	82
B	Skeletal muscle	1.13 ± 0.25	1.28 ± 0.21	2.87 ± 0.83	2.78 ± 1.1	0.71 ± 0.24	0.05 ± 0.01	<0.01	n.e.	27
Pr	Skeletal muscle	3.1 ± 0.77	4.1 ± 2.4	1.45 ± 0.41	n.e.	0.43 ± 0.30	0.03 ± 0.01	<0.02	n.e.	31

[a] n.e. = not examined.

hindrance to the trimethylsilyl group. If the 16-methyl group is *trans* to the hydroxyl group this does not obtain. In the case of triamcinolone the two hydroxyl groups are also in the *cis* configuration but the 16-hydroxyl group provides less steric hindrance to the silylation of the 17-hydroxyl group because it is smaller than the methyl group of dexamethasone. This is presumably the reason for the stability of the triamcinolone derivative on GLC. The observation that the triamcinolone acetonide derivative is stable on GLC although the C_{20} ketone is not silylated may be explained on the grounds that steric hindrance prevents both the formation of the trimethylsilyl ether of the ketone (via the enol form) and reaction between this group and the column materials. This factor would prevent adsorption and consequent tailing on the column as well as breakdown of the derivative due to loss of the side-chain. It seems likely that it is the esterification of the enolised ketone groups of the corticosteroids which results in the breakdown of the compounds during attempts at purification, as trimethylsilyl ethers of secondary alcohols are usually stable. This instability has been noted by WOTIZ AND CLARK[15].

The basis of the electron-capture detector response to the corticosteroid derivatives is not fully understood. The response appears to depend on the presence of the conjugated system in the corticosteroids together with the oxygen functions in Ring D and the dihydroxyacetone side-chain. The single fluorine atom in triamcinolone, triamcinolone acetonide, dexamethasone and betamethasone does not significantly increase the response of the detector. The presence of an oxygen function at C_{17} and of a substituent in the cyclopentane ring seems to be of greater importance since the response of the detector to corticosterone is less than 30 % of its response to cortisol, and the response to prednisolone and cortisol is lower than that to betamethasone, triamcinolone and triamcinolone acetonide.

The sensitivity of the electron-capture detector to steroid molecules in which there is either conjugation, or a number of closely positioned oxygen atoms, has been used by other investigators. RAPP AND EIK-NES[16] devised a method for the estimation of aldosterone by the formation of its γ-lactone. This form was found to be more electron-capturing than the chloroacetate of aldosterone. DE JONG AND VAN DER MOLEN[17] used GLC with electron-capture detection for the measurement of dehydroepiandrosterone levels in plasma. The compound was oxidised with chromium trioxide to androst-4-ene-3,6,17-trione. The smallest amount detectable by the electron-capture detector used was 0.1 ng. This is similar to the minimum quantity of the trimethylsilyl ethers of the corticosteroids detected by the method described herein, and to that of the monochloroacetate of progesterone in the method developed by VAN DER MOLEN AND GROEN[18].

The estimation of endogenous cortisol in mammalian tissues by the method described cannot be recommended as the physiological concentrations of these steroids are low and the use of competitive protein-binding radioassay would provide greater sensitivity and specificity[19,20]. However, the method can be used for the estimation of synthetic glucocorticosteroids: it is relatively simple and the specific electron-capturing properties of the corticosteroid structure mean that there is no interference from normal tissue components which might be extracted simultaneously with the steroids.

Improvement in the sensitivity of the method could be achieved by the use of a multi-port switching valve with a short pre-column, at the head of the main

column, in order to direct the carrier gas, with most of the solvent, away from the detector for the first few minutes after injection. During this period pure carrier gas from another column would be directed through the detector in order to maintain the normal standing current. This device would eliminate the large solvent peak and consequently raised baseline which is responsible for some loss in sensitivity of the detector, as shown by RAPP AND EIK-NES[21]. Using the switching-valve it would be possible to inject larger quantities of samples with lower concentrations of steroid, thus lowering the detection limit of the method.

The results presented show that the present sensitivity of the method is sufficient to allow the estimation of the corticosteroids in muscle for a period of several hours, and in the case of triamcinolone acetonide, 24 h, after intraperitoneal administration of a dose of 20 mg/kg to rats.

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