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Note

Extraction and separation of androstenedione from products of aromatase assays on micro-columns of magnesium oxide

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Transformations of steroid hormones, particularly the aromatization of androgens, are being examined with increasing frequency in central neural tissues [e.g. ref. 1].

While many extraction procedures are available, two commonly encountered problems are low recoveries of steroids and the presence of unwanted lipid which must usually be removed before further analysis. Solvent and phenolic partitioning have been widely used to extract lipid contaminants and unreacted substrate in aromatase reactions [2].

The procedure described, in conjunction with a modification of the magnesium oxide micro-column method of Kawahara et al. [3], gives greater recoveries of steroids with less contaminating lipid, and permits rapid and efficient separation of unreacted substrate from products of aromatase assays with neural tissue, cultured cell suspensions and microsomal fractions of human placenta.

MATERIALS AND METHODS

Water was glass-distilled. All solvents were redistilled before use. Unlabelled steroids were obtained from Sigma (St. Louis, MO, U.S.A.). The following radiolabelled steroids were obtained from Amersham (Australia): [4-^{14}C]-androstenedione (58 mCi/mmol), [1,2,6,7-^{3}H]-testosterone (83 Ci/mmol), [2,4,6,7-^{3}H]-oestrone (92 Ci/mmol), [2,4,6,7-^{3}H]-17β-oestradiol (92 Ci/mmol) and [2,4,6,9-^{3}H]-oestriol (92 Ci/mmol)*. These were checked for homogeneity

*Androstenedione = 4-androsten-3,17-dione; testosterone = 17β-hydroxy-4-androsten-3-one; oestrone = 3-hydroxy-1,3,5[10]-oestratrien-17-one; 17β-oestradiol = 1,3,5[10]-oestratriene-3,17β-diol; oestriol = 1,3,5[10]-oestratriene-3,16α,17β-triol.

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before use by thin-layer chromatography in the following systems by volume; benzene—toluene—methanol (9 : 1 : 1) and diethyl ether—benzene (2 : 1).

Magnesium oxide (light) was obtained from BDH (Poole, U.K.) and was washed extensively with water to remove fines, then heated overnight at 180°C before use.

Fresh lamb brains were obtained from Westfield Freezing Works (Auckland, New Zealand). The hypothalami were excised and homogenised with an Ultra Turrax instrument at full speed for 15 sec in phosphate buffered saline (PBS): 10 m*M* sodium phosphate—0.15 *M* sodium chloride, pH 7.4 (1 : 3, w/v).

Androstenedione and oestrone were chosen as representative C_{19}- and C_{18}-steroids respectively and were used in recovery experiments. Labelled steroids were added to 25-ml conical flasks and the solvent evaporated under a fine stream of oxygen-free nitrogen. Just prior to the addition of homogenate, the steroids were redissolved in 4 drops of absolute ethanol. A 5-ml sample of homogenate was added, mixed and immediately frozen in a mixture of dry-ice and ethanol. After thawing, 25 μg each of unlabelled androstenedione and oestrone, in 0.5 ml ethyl acetate was added and the mixture extracted with 4 volumes of 2,2,4-trimethylpentane (isooctane)—ethyl acetate (93 : 7, v/v) four times. Extracts were dried under reduced pressure, the volume made to 1 ml with ethyl acetate and duplicate samples taken for liquid scintillation counting. A sample was also taken for total lipid estimation [4].

The micro-column method of Kawahara et al. [3] was modified to allow separation of androstenedione from products of aromatization, with a single change of solvent. Disposable plastic insulin syringes (1 ml) were used as columns and were plugged with a small piece of glass wool. A slurry of magnesium oxide and isooctane was poured and allowed to settle by gravity to a height of 3 cm. Approximately 5 ml isooctane was then run through the column to ensure uniform packing. Samples were dissolved in 50—75 μl ethylmethylketone to which was added 0.4 ml isooctane and the sample was layered onto the column. Sample tubes were then rinsed with 0.15 ml isooctane twice and both washes were applied to the column. Elution was begun with 10 ml isooctane—ethyl acetate (9 : 1, v/v) then with 6 ml ethyl acetate—methanol (8 : 2, v/v); each fraction was collected separately. Fractions were dried under nitrogen, made up to 1 ml with ethyl acetate and samples were taken in duplicate for liquid scintillation counting.

For phenolic partitioning, steroids were extracted from neural tissue homogenates, partitioned against sodium hydroxide and re-extracted with diethyl ether, essentially as described by Ryan [2].

Scintillation counting was performed using a Packard Tricarb 3200 Liquid Scintillation Spectrometer, with scintillation fluid containing 0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis(5-phenyloxazolyl-2)benzene in toluene. Results were analysed according to the equations of Coghlan et al. [5] for double-label experiments.

RESULTS AND DISCUSSION

On the basis of experiments with androstenedione and oestrone, the extraction procedure described was compared to three previously published

TABLE I

COMPARISON OF METHODS FOR EXTRACTION OF [^{14}C]-ANDROSTENEDIONE AND [^{3}H]-OESTRONE FROM NEURAL TISSUE HOMOGENATES

Steroids were extracted from lamb brain homogenates by each of the techniques described, dried under reduced pressure, made up to 1 ml ethyl acetate and samples taken for liquid scintillation counting and total lipid estimation.

Extraction method [ref.]	Steroid recovered (%) (± S.D.)*		Lipid extracted (mg) (± S.D.)*
	[^{14}C]-Androstenedione	[^{3}H]-Oestrone	
5 vol. Chloroform, three times [2]	63.85 (1.8)	64.4 (2.5)	27.2 (1.7)
5 vol. Water saturated ethyl acetate, three times [8]	82.5 (4.4)	87.9 (3.7)	37.5 (0.9)
5 vol. Diethyl ether, three times [9]	75.9 (3.1)	88 (2.4)	34.9 (2.5)
4 vol. Isooctane—ethyl acetate (93:7, v/v) four times	96.9 (2.2)	96.3 (4.8)	7.9 (0.6)

*S.D. = standard deviation (n = 3).

methods with respect to recovery of steroids and the amount of contaminating lipid in the extract. These data are presented in Table I.

Extraction of steroids from homogenates of neural tissue with isooctane—ethyl acetate gives significantly higher recoveries, with much less lipid, about 25% of that found with the other procedures. In addition, two further advantages are apparent: (a) the solvent did not readily form emulsions with the aqueous phase, thereby obviating the need for centrifugation which is frequently necessary where ethyl acetate is used and (b) the immiscibility of the organic and aqueous phases makes it unnecessary to dry organic extracts over such compounds as anhydrous sodium sulfate [6]. The method has been applied by the authors to the extraction of steroids from aromatase assays using cell suspensions and human placental microsomal preparations. With [4-^{14}C]-androstenedione as substrate, the average recovery of label was 92 ± 4% (n = 29).

When the various extracts were applied to micro-columns of magnesium oxide only the isooctane—ethyl acetate extracts could be eluted, in contrast to the other extracts which caused the columns to clog.

Table II compares total recoveries and separation of [^{14}C]-androstenedione and [^{3}H]-oestrone after extraction from brain homogenates with isooctane—ethyl acetate, followed by either magnesium oxide micro-column chromatography or phenolic partitioning. The results indicate that both total recovery and overlap of steroids in the various fractions are more efficient with magnesium oxide micro-columns than with phenolic partitioning. It is worthwhile noting that the magnesium oxide micro-column method requires only 2—3 h to complete, using much less solvent and fewer manipulations than phenolic partitioning.

TABLE II

SEPARATION OF [^{14}C]-ANDROSTENEDIONE AND [^{3}H]-OESTRONE EXTRACTED FROM BRAIN HOMOGENATES BY MAGNESIUM OXIDE MICRO-COLUMNS AND PHENOLIC PARTITIONING

Steroids were extracted and magnesium oxide micro-columns run as described under Materials and methods. Phenolic partitioning of steroids was performed according to the method of Ryan [2].

Technique	Steroid recovered (%) (± S.D.)*	
	[^{14}C]-Androstenedione	[^{3}H]-Oestrone
Magnesium oxide micro-column:		
Isooctane—ethyl acetate	91.7 (2.2)	2.8 (0.5)
Ethyl acetate—methanol	4.2 (0.8)	86.8 (3.0)
Phenolic partitioning:		
Androgen fraction	89.8 (1.2)	6.4 (4.6)
Phenolic fraction	1.2 (1.0)	75.2 (1.5)

*S.D. = Standard deviation (n = 4).

As the authors are concerned with enzymic transformations of androstenedione by various cell lines (unpublished data), it was of interest to test the suitability of the magnesium oxide micro-column method for the separation of unreacted androstenedione from testosterone and oestrogens other than oestrone. For this purpose, 25 μg each of androstenedione, testosterone, oestrone, 17β-oestradiol and oestriol were added to test tubes containing ^{14}C-labelled androstenedione and tritiated testosterone, oestrone, 17β-oestradiol and oestriol. Columns were run and fractions analysed for total [^{14}C] and [^{3}H]; these data are presented in Table III.

The results show that this method offers advantages for two main reasons: (a) it allows rapid and efficient separation of unreacted substrate from products, which is especially important in systems where conversion is low, making subsequent characterization of products by chromatography or recrystallization easier, and (b) it allows retention of products which might

TABLE III

SEPARATION OF [^{14}C]-ANDROSTENEDIONE FROM [^{3}H]-TESTOSTERONE, [^{3}H]-OESTRONE, [^{3}H]-17β-OESTRADIOL AND [^{3}H]-OESTRIOL ON MAGNESIUM OXIDE MICRO-COLUMNS

A 25-μg amount each of unlabelled androstenedione, testosterone, oestrone, 17β-oestradiol and oestriol was added to test tubes containing [^{14}C]-androstenedione (99,500 dpm) and [^{3}H]-testosterone, [^{3}H]-oestrone, [^{3}H]-17β-oestradiol and [^{3}H]-oestriol (total [^{3}H] = 572,900 dpm). Columns were run and results analysed as described under Materials and methods and are presented as total [^{14}C] and [^{3}H] recovered.

Column fraction	[^{14}C] Recovered (%) (± S.D.)*	[^{3}H] Recovered (%) (± S.D.)*
Isooctane—ethyl acetate	96.3 (4.8)	2.4 (0.3)
Ethyl acetate—methanol	5.6 (0.8)	98.3 (4.5)

*S.D. = Standard deviation (n = 6).

otherwise be lost when liquid—liquid partition systems are employed. In addition, the method as described results in an increase in recovery of steroids of approximately 20% over the original method of Kawahara et al. [3] and from 10—20% over the Sephadex LH-20 micro-column method of Gips et al. [7].

The authors believe the two procedures described allow more efficient extraction and separation of C_{19}- and C_{18}-steroids from experiments using neural tissues, where samples have a high lipid content.

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