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Determination of remoxipride in plasma and urine by reversed-phase column liquid chromatography

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SUMMARY

A reversed-phase column liquid chromatographic method for the determination of remoxipride, a novel antipsychotic drug, in biological fluids is described. A simple one-step extraction is used followed by liquid chromatography on a 3-μm octadecylsilica column and ultraviolet absorbance detection. The method is accurate and precise for clinical remoxipride levels in both plasma and urine. For situations where a higher sensitivity is necessary a two-step extraction and a modified mobile phase are used. With this modification plasma concentrations down to 2 nM can be determined with acceptable precision.

INTRODUCTION

Remoxipride, a substituted benzamide, has been shown to be a selective dopamine-D_2 receptor antagonist [1], a property shown to be closely connected to the antipsychotic effect of neuroleptic drugs [2]. Remoxipride has very little affinity for other receptors [3], suggesting that the compound is less likely than classical neuroleptics to produce extrapyramidal symptoms and other side-effects. Clinical trials have confirmed that remoxipride has antischizophrenic effects with few extrapyramidal or other adverse effects reported [4–8].

So far, only one report on the analysis of remoxipride in biological fluids has been published [9]. This method utilized reversed-phase liquid chromatography (LC) with direct injection of the sample into a precolumn loaded with

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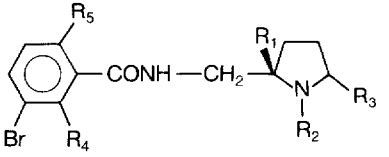
hexadecyltrimethylammonium bromide. The compounds were detected with fluorescence using a rather complicated post-column extraction system, in which remoxipride was extracted as an ion-pair with a highly fluorescent counter-ion, 9,10-dimethoxyanthracene-2-sulphonate. In plasma, a concentration of 3 n*M* gave a signal-to-noise ratio of 3:1. The urine determinations were hampered by endogenous interferences, giving impaired sensitivity (40 n*M* could be detected) and reproducibility. Some reports on the analysis of remoxipride metabolites have also been published [10,11].

This paper describes a simple and robust reversed-phase LC method for the routine determinations of remoxipride in plasma and urine. Clinical levels can be determined with very good precision and accuracy. For pharmacokinetic and pharmacological studies, where a high sensitivity is often valuable, a modification of the method is presented. This modification gave a limit of quantification of 2 n*M*.

EXPERIMENTAL

Chemicals and reagents

Remoxipride, (−)-(*S*)-3-bromo-N-[(1-ethyl-2-pyrrolidinyl)methyl]-2,6-dimethoxybenzamide, the internal standard (I), 3-bromo-N-[(1-propyl-2-pyrrolidinyl)methyl]-2,6-dimethoxybenzamide, and the metabolites (II–V) were prepared at CNS Research and Development, Astra Research Centre (Södertälje, Sweden). The molecular structures are shown in Fig. 1. N,N-Dimethyl-N-octylamine (DMOA) and N,N-dimethyl-N-nonylamine (DMNA)



	R_1	R_2	R_3	R_4	R_5	
remoxipride	H	C_2H_5	2H	OCH_3	OCH_3	
I (Internal standard)	H	C_3H_7	2H	OCH_3	OCH_3	(racemic)
II	H	C_2H_5	2H	OH	OCH_3	
III	H	C_2H_5	2H	OCH_3	OH	
IV	OH	C_2H_5	=O	OCH_3	OCH_3	
V	OH	H	=O	OCH_3	OCH_3	

Fig. 1. Structures of remoxipride, internal standard (I) and metabolites (II–V)

were purchased from ICN Biomedicals (Plainview, NY, U.S.A.) and decyl sodium sulphate from Research Plus (Bayonne and Denville, NJ, U.S.A.). Other chemicals were of analytical or HPLC grade, obtained from the usual commercial sources and used without further purification. Standard solutions of remoxipride and I were prepared in phosphate buffer (0.05 *M* sodium dihydrogen orthophosphate adjusted to pH 2 with orthophosphoric acid) and stored in a refrigerator. New standard solutions were prepared every second month.

Extraction

Depending on the expected concentrations, two slightly different procedures were used.

Procedure I (normal concentrations). To each sample tube containing 0.50 ml of plasma or urine, an appropriate amount of I was added followed by 0.5 ml of 0.2 *M* sodium hydroxide solution and 4 ml of *n*-hexane–diethyl ether (20:80, v/v). The tubes were slowly rotated in a rotary mixer for 10 min and were then centrifuged for 5 min at 1500 *g*. The organic phase was transferred to another tube and evaporated under a stream of nitrogen at 40°C. The residue was reconstituted in 0.5–3 ml of phosphate buffer (pH 2), the volume depending on the expected concentration, and 75 μl were injected.

Procedure II (low concentrations). To each sample tube, containing 1.00 ml of plasma, an appropriate amount of I was added followed by 0.5 ml of 1 *M* sodium hydroxide solution and 4 ml of diethyl ether–*n*-heptane (30:70, v/v). This extraction often resulted in emulsion formation, but this emulsion could be broken by freezing the samples for 1 h at −20°C. After centrifugation and evaporation as above, the residue was dissolved in 300 μl of phosphate buffer (pH 2). Thereafter, 750 μl of the organic phase were added. After a short (10 s) mixing on a vortex mixer, the aqueous phase was transferred to the autosampler vial and 200 μl were injected.

To study the absolute recoveries of remoxipride and the internal standard from plasma, urine and water, extractions were performed using aliquots. The absolute recovery was calculated by dividing the slope of the concentration versus peak-height graph by the slope of a calibration graph obtained from direct injection of aqueous standard solutions of the compounds.

Liquid chromatography

The solvent-delivery system was an LKB 2150 pump. The autosampler was a Perkin-Elmer ISS-100 equipped with a 200-μl sample loop and an external by-pass loop (500 mm×0.125 mm I.D.) connecting the valve inlet and outlet capillaries. Chromatography was performed on a column (100 mm×4.6 mm I.D.) factory-packed with 3-μm octadecylsilica particles (Nucleosil 120-3 C_{18}) from Macherey & Nagel (Düren, F.R.G.). The UV absorbance detector was a Perkin-Elmer LC-95 with a 4.5-μl cell volume (response time 0.1 s) or a Spectra-Physics SP8450 with a 14-μl cell volume (response time 0.5 s). Both de-

tectors were operated at 206 nm. The second detector was preferred for the high-sensitivity determinations as the filter used made it possible to use a higher response time and, together with the larger cell volume, a four-fold improvement in signal-to-noise ratio was achieved. The drawback was a small decrease, ca. 10%, in the chromatographic efficiency.

The mobile phase, degassed with helium before use, was acetonitrile–phosphate buffer (pH 2) (25:75, v/v) with 0.2 m*M* DMNA (procedure I). For the low concentration determinations (procedure II) the mobile phase was modified to acetonitrile–phosphate buffer (pH 2) (30:70, v/v) with 0.4 m*M* DMOA and 0.5 m*M* decyl sulphate. The flow-rate was 1.3 ml/min.

A Spectra-Physics SP4270 integrator was used to measure the peak heights and calculate the peak-height ratio of remoxipride versus I. The unknown concentrations were calculated from a two-point calibration graph, the first point being the origin, confirmed each day of analysis, and the second point the mean peak-height ratio for four to eight standard samples (drug-free plasma spiked with the same remoxipride concentration).

Stability

Remoxipride was added to glass test-tubes containing 0.50 ml of plasma, giving a concentration of 0.5 μM, or to glass or polypropylene test-tubes with 1.00 ml of urine, giving concentrations of 1.0 or 5.0 μM. The tubes were stored under the following conditions: (i) At room temperature for 48 h, exposed to daylight; (ii) at 6°C (refrigerator) for two weeks; (iii) at −20°C (freezer) for ca. fifteen months; (iv) at −70°C for fifteen months (only urine in polypropylene tubes). For each storage condition, samples were taken five to seven times, three or four samples each time.

Validation

The precision and accuracy were studied by spiking a number of drug-free plasma samples with known amounts of remoxipride followed by analysis according to the method described above.

Control samples (drug-free plasma or urine spiked with remoxipride) were made in batches of ca. fifty samples, with concentration levels appropriate for the samples to be analysed. The control samples were stored frozen. Three control samples were analysed together with each batch of unknown samples.

RESULTS AND DISCUSSION

Extraction

Extraction is still the most used sample work-up procedure for the assay of drugs in biological fluids. The advantages are an efficient clean-up of the sample and that the sample can be concentrated, allowing improved sensitivity.

The main disadvantage is that the extraction step is time-consuming and therefore often the capacity-limiting step.

In a previous method [12], deproteinized plasma was injected into a coupled-column system. The simplified sample work-up permitted 40–50 samples to be prepared in 90 min. However, problems with large batch variations for both column packings, necessitating time-consuming method modifications, made it less suitable for routine use.

Remoxipride is a tertiary amine with an acid dissociation constant of 8.9, and the method described in this paper used liquid–liquid extraction at alkaline pH for the sample work-up. A simple one-step extraction was found to be sufficient to provide both acceptable drug-free plasma chromatograms (Fig. 2) and quantitative recovery of remoxipride and I (Table I). The work-up time for 40–50 samples was in this case 4 h. If remoxipride concentrations in the low nanomolar range were to be determined, the chromatograms were not clean enough. An alternative extraction with a modified organic phase and an additional clean-up step was tried. The same extraction was earlier used for the determination of haloperidol in plasma [13]. This extraction, combined with a modified mobile phase (procedure II), gave chromatograms without inter-

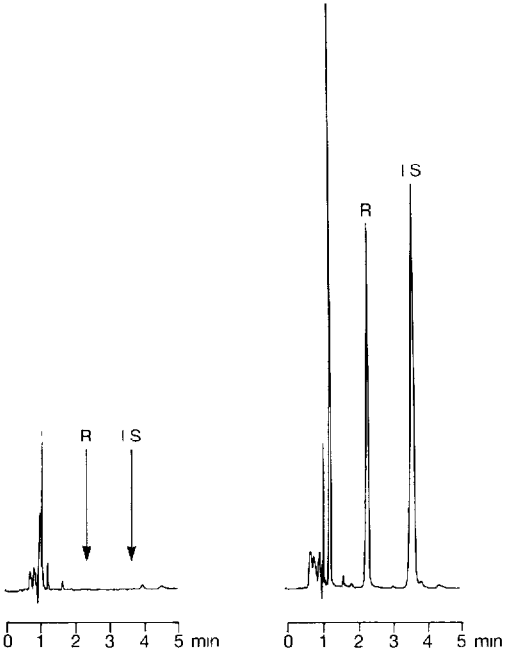


Fig. 2. Human plasma chromatograms for procedure I: drug-free plasma (left) and 6 h after an oral 100-mg dose (right). The after-dose remoxipride concentration was found to be 2.49 μM and the added concentration of the internal standard was 4.00 μM. R and I.S. indicate the positions of remoxipride and the internal standard, respectively.

TABLE I

ABSOLUTE RECOVERIES

Compound	Medium	Procedure	Absolute recovery (%)
Remoxipride	Plasma	I	100
	Urine	I	99
	Water	I	100
	Plasma	II	85
Internal standard	Plasma	I	102
	Urine	I	101
	Water	I	102
	Plasma	II	95

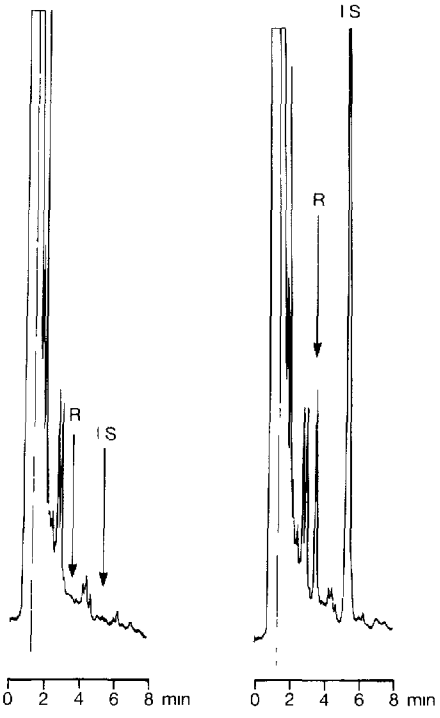


Fig. 3. Rat plasma chromatograms for procedure II: drug-free plasma (left) and 30 min after an intraperitoneal 1 μmol/kg dose (right). The after-dose remoxipride concentration was found to be 20 n*M* and the added concentration of the internal standard was 100 n*M* R and I.S indicate the positions of remoxipride and the internal standard, respectively.

fering endogenous compounds and the high sensitivity of the UV absorbance detector could be better utilized (Fig. 3). The absolute recovery was almost quantitative for I but decreased to 85% for remoxipride (Table I). The somewhat low recovery did not, however, influence the precision. The work-up time for 40–50 samples was 6 h.

Column liquid chromatography

An amine (DMOA or DMNA) was added to the mobile phase to improve peak symmetry [14]. Since it had the same charge as remoxipride, it also worked as a competing ion, i.e. an increasing concentration of amine in the mobile phase decreased the capacity factor for remoxipride and I. The addition of an anion, decyl sulphate, increased the capacity factor for both compounds. As both these additives changed the retention for amines rather selectively, they could be used to regulate the selectivity, which was beneficial for the low-concentration determinations.

The mobile phases were always recirculated. In this way the mobile phase was saturated with dissolved silica, which improved the stability of the column [15]. Besides the improvement in stability, consumption of the solvent and reagent was decreased. The lifetime of the columns was typically more than six months. The contamination of the mobile phase with analyte and endogenous components gradually increased the background signal, but normally the same mobile phase could be used for about a week or for 250 samples.

By-pass capillary

Autosamplers with electrically actuated valves were recently introduced. These valves are more practical but give longer switching times than pneumatically actuated valves owing to the less powerful actuation. In our study, the combination of a constant-flow pump with fast electronic flow compensation and an autosampler with an electrically actuated valve gave a decrease in the column efficiency during long runs, owing to the formation of a barely noticeable void at the column top. The void was a result of the fast pressure build-up (from 120 to 200 bar) when the flow is blocked during the switching movement. The pressure is then relaxed as a pulse hitting the column top. The column performance was always restored by repacking of the column top. Figure 4 illustrates the efficiency decrease during a run with 108 extracted plasma samples. As the chromatographic efficiencies of remoxipride and I were not affected to the same extent, the peak-height ratio was also changed (Fig. 5), giving incorrect results.

To avoid the pressure pulse and improve the stability of the column, a by-pass capillary was installed connecting the valve inlet and outlet capillaries [16]. The difference in flow resistance between the by-pass capillary and the injection loop must be so large that the eluent always flows through the loop except when the valve is switched. During this short time, the eluent flows

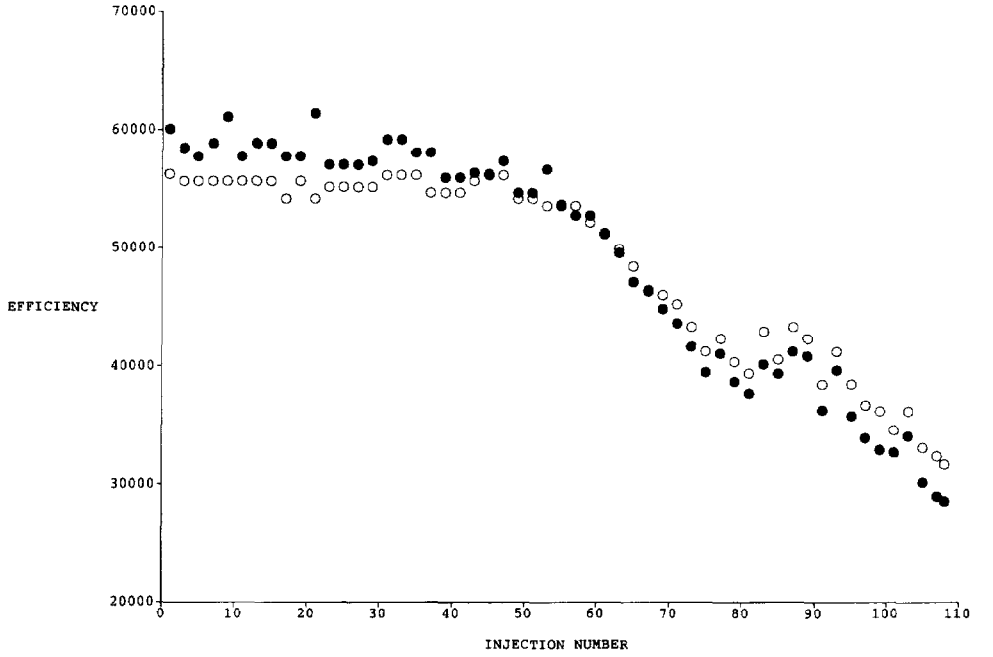


Fig. 4 Column efficiency decrease during a long run without the by-pass capillary: (○) remoxipride; (●) internal standard. The efficiency is given as N/m.

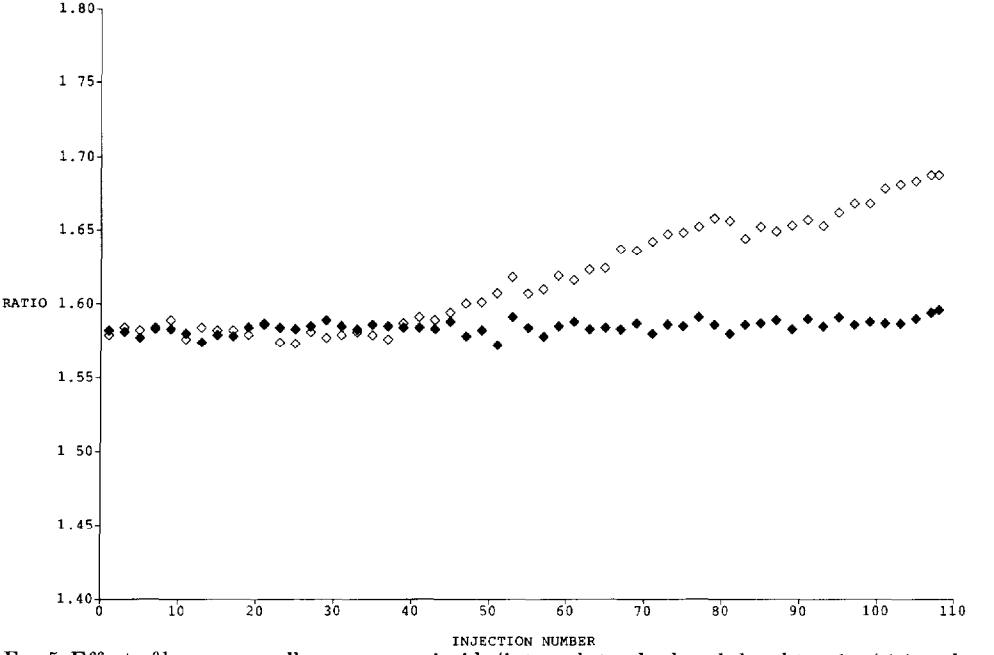


Fig 5. Effect of by-pass capillary on remoxipride/internal standard peak-height ratio· (◇) without by-pass capillary; (◆) with by-pass capillary.

through the by-pass capillary. The small difference in flow resistance between the sample loop and the by-pass capillary will not give any significant pressure pulses. The experiment above was repeated with the by-pass capillary installed. No loss in efficiency was observed and, consequently, the peak-height ratio was constant (Fig. 5).

Selectivity

Remoxipride is metabolized to a great extent both in animals and humans [17]. It is, consequently, important to study whether the metabolites might interfere with the remoxipride assay. The metabolites published so far are the phenolic compounds III [10] and II (tentative) [11] and the hydroxylactams IV and V [18] (the structures are shown in Fig. 1). Table II lists the retention times for these metabolites, together with their absolute recoveries, in the two procedures for remoxipride. In procedure I, V eluted close to remoxipride and III eluted close to I. However, considering the low recovery of V and the very low levels of III [10], it can be concluded that these metabolites will not interfere in the remoxipride assay.

Some other metabolites, not yet published [17], were also tested in the same way and found not to interfere.

No interfering peaks from concurrent medication were observed using procedure I. However, occasionally peaks of endogenous origin interfered and the mobile phase had to be modified. The best way was usually to change the amine modifier or its concentration, or to add a counter-ion, e.g. octyl sulphate or nonyl sulphate. These additives selectively change the retention of remoxipride and I towards compounds without charge at the mobile phase pH. Consequently, the selectivity against the lactam (cyclic amide) metabolites, where the basicity of the amine is lost, was changed (see Table II) and the additives therefore must be used with some caution. The selectivity of procedure II against other drugs and occasional endogenous peaks was not as thoroughly studied since this procedure was used in only a few pharmacokinetic studies in animals.

TABLE II

SELECTIVITY

Compound	Retention time (min)		Absolute recovery (%)		Amine/lactam
	Procedure I	Procedure II	Procedure I	Procedure II	
Remoxipride	2.35	3.37	100	85	Amine
I	3.60	5.27	102	95	Amine
II	6.25	9.51	50	<1	Amine
III	3 73	5 99	60	2	Amine
IV	4 05	1.99	3	<1	Lactam
V	2.50	1.45	<1	<1	Lactam

Sensitivity, repeatability and linearity

The instrumental limit of detection, defined in ref. 13, was 100 fmol. The limit of quantification, when procedure II was used, was 2 n*M*. This concentration corresponds to an injected amount of about 1 pmol. A vast majority of the samples to be assayed were human plasma samples from clinical trials giving rather high remoxipride levels (0.5–10 μM). In these cases, it was not necessary to report levels below 50 n*M*, and procedure I was adequate. Procedure I could be used to assay concentrations down to 20 m*M*, but for pharmacological and pharmacokinetic studies in humans and animals the higher sensitivity of procedure II was sometimes needed. The within-run precision and accuracy for both procedures are presented in Table III.

The analysis of control samples showed that the between-run precision was excellent. The coefficients of variation (C.V.) for some batches of control samples are shown in Table IV.

As described above, a two-point calibration graph was used for calculating the unknown concentrations. This graph can be used only if the linearity has been confirmed during the method validation work. Non-linearity might occur as a result of overloading of the chromatographic column or of exceeding the

TABLE III

WITHIN-RUN ACCURACY AND PRECISION IN PLASMA

Procedure	Accuracy		Precision, C.V (%)	*n*
	Added concentration (n*M*)	Found concentration (mean) (n*M*)		
I	200	203	1.0	12
	2000	2000	0.9	12
II	2.0	1.9	5.2	10
	20.0	19.5	2.2	10

TABLE IV

BETWEEN-RUN PRECISION FOR CONTROL SAMPLES

Analysis according to procedure I.

Concentration (μM)	Biological fluid	C.V. (%)	*n*
0.2	Plasma	4.0	20
5	Plasma	3.7	12
5	Plasma	2.7	23
1	Urine	2.3	15
10	Urine	3.1	16

linear range of the detector. For this method overloading was no problem, but the rather high straylight of the UV absorbance detectors caused non-linearity at injected amounts above 2000 pmol. The amounts injected during analysis were never greater than 500 pmol as the samples were always diluted according to the expected concentrations. Calibration graphs with eight to ten different concentrations have been analysed on several occasions. No deviations from linearity were observed, and the correlation coefficients were 0.9990 or better.

Stability

In plasma, remoxipride was found to be stable under all investigated conditions (Table V). In urine, a decrease was seen in the samples stored frozen for a long time. This decrease was, however, less than 5% and only slightly greater than the C.V. for control samples in urine (see above). Although the decrease is uncertain, analysis of urine samples within six months is recommended. Remoxipride was stable in urine under the other storage conditions investigated (Table V).

Experience of the method in routine use

So far ca. 30 000 samples have been assayed with this method (e.g. ref. 19). The samples were mainly human plasma, but also other biological materials, such as urine, blood and tissue homogenates from humans and animals, with concentrations ranging from 2 n*M* to 700 μM. The chromatographic system is simple and requires a minimum of maintenance. Changing the mobile phase once a week and repacking the column top once or twice a month is usually sufficient. Very few problems with interfering peaks have been seen. The method is thus well suited for routine use.

TABLE V

STABILITY OF REMOXIPRIDE IN PLASMA AND URINE

Sample	Tube material	Temperature (°C)	Time	Total decrease (%)
Plasma	Glass	20	48 h	0
	Glass	4	2 weeks	0
	Glass	−20	13.5 months	1
Urine	Glass	20	48 h	0
	Glass	4	2 weeks	1
	Glass	−20	15 months	3.5
	Polyprop.	−20	15 months	4.5
	Polyprop.	−70	15 months	4.5

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